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EMBRYOLOGIA, Vol. 5, No. 2, pp. 127–138 (July, 1960)

ANALYSIS OF GENE ACTION IN THE TRANSPARENT-SCALED GOLDFISH, *CARASSIUS AURATUS*

II. THE EFFECTS OF PITUITARY AND THYROID ON GENE ACTION

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The larval chromatophores (melanophores, xanthophores and guanophores) of the homozygous transparent-scaled goldfish undergo degeneration about ten days after hatching, when the larvae reach a length of 6 to 7 mm. In the previous experiments (KAJISHIMA, '60) it has been indicated that this racially specific behavior is determined by the genetical characters of the chromatophores themselves, while the time of gene action is controlled by factor (or factors) external to the chromatophores.

In recent years voluminous literature on the hormonal control of chromatophores by the hypophysis has been accumulated. Basing upon the experiments of hypophysectomy in amphibian embryos, it has been shown by ALLEN ('16), SMITH ('16), SWINGLE ('21), ATWELL ('36), DRISCOLL and EAKIN ('55) and BAGNARA ('58) that the intermediate lobe of the hypophysis produces a hormone or hormones, which not only stimulate chromatophores to expand or contract, but also promote synthesis and destruction of the pigment granules.

In teleostean fishes it has been generally believed that the control of melanophores is chiefly mediated by the sympathetic nervous system whereas the hormonal control is not so evident as in amphibians. However, OSBORN ('41 a, b) and CHAVIN ('56, '59) have found that the melanogenesis in fishes does not occur after hypophysectomy. In regard to xanthophores, it is well-known that in various fishes the hormone of the pars intermedia elicits dispersion of the pigment granules (cf. PARKER, '49). Recently in the xanthic goldfish, CHAVIN ('56) has also observed the degeneration of lipo-

phores (xanthophores and erythrophores) after hypophysectomy.

On the other hand, an effect of the thyroid gland on the pigmentation of the teleostean fish has been suggested by MARINE and LENHART ('10) in some trout. TERAO ('22) and BLACHER ('27) have found the decolouration of young goldfish is promoted by the thyroid feeding. In the particoloured (black-red or black-yellow) goldfish MÜLLER ('53) has also reported the melanophore dispersion and the promotion of colour change after prolonged injection of thyroxine. Recently HONMA and MURAKAWA ('57) have observed the retardation of decolouration in the young goldfish by the treatment with an anti-thyroid drug.

From these results it seems possible that the chromatophore-degeneration in the transparent type is controlled by the pituitary or/and thyroid hormones. The present paper deals chiefly with the effects of the pituitary and thyroid upon the gene action which is directly responsible for the chromatophore-degeneration. Also some observations have been made on developmental changes of these endocrine organs in the course of the chromatophore-degeneration.

MATERIAL AND METHOD

The goldfish used was the inbred offspring of the homozygous transparent type (*TT*), which had been segregated by self-breeding of the heterozygous fish of the race. The offspring of the homozygous normal type (*tt*) which had been inbred also from the same heterozygous type was used as control.

To analyse the hypophysial effect on the gene action, the presumptive hypophysis was removed from tail-bud embryos. In this stage, hypophysial placode lies between the telencephalon and the yolk mass (Fig. 1). The embryo was anaesthetized in a 1/10 saturated chloretone solution for about 10 minutes, and the hypophysial primordium together with the surface ectoderm was cut off with a fine steel needle. Embryos thus operated were transferred to double-strength HOLTFRETER's solution for about 2 to 3 days and then to ordinary tap water.

The inhibition experiment of the thyroid hormone was performed by using phenylthiourea. About 50 eggs were immersed in 1 litre of 0.05% solution of this drug soon after spawning. Half of the

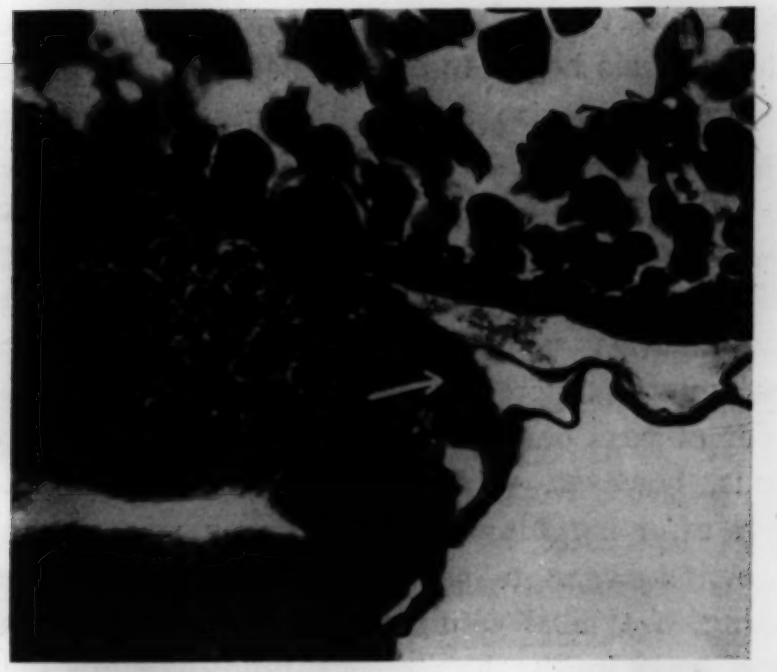

Fig. 1. Longitudinal section through the primordium of the hypophysis (arrow) in the tail-bud embryo of goldfish. ×400

solution was changed twice a week. The larvae were fed first with *Artemia* and later with *Tubifex.*

For the histological investigations, fish were fixed in BOUIN's solution and serially sectioned in paraffin at 8 to 10 μ. thickness. For the staining, HEIDENHAIN's azan and GOMORI's chrome-alum hematoxylin were used.

RESULTS

Normal development of pituitary and thyroid glands: The hypophysial primordium first appeared in the tail-bud stage (about 40 hours after spawning) as a strand of cells extending inward from the inner layer of the surface ectoderm below the telencephalon. At the time of hatching, the cell mass invaginated posteriorly and formed a definite mass lying between the endoderm and the infundibulum, just at the tip of the notochord. The hypophysial rudiment seemed to be consisting of cells which belonged to the same cell type as the cells of undifferentiated ectoderm. The rudiment was separated into two parts by a cleft. On the 4th day after hatching, it fused with the floor of the infundibulum, forming

the fibrous pars nervosa in the central portion. The cells of the epithelial hypophysis began to differentiate. In the anterior portion the cells increased in size and in the posterior region the cells began to become basophilic. Ten days after hatching, a mass of large cells which were faintly basophilic became noticeable in the transitional region, and a granular substance appeared in the pars nervosa. This substance was markedly stained with chrome-alum hematoxylin and was apparently the neurosecretory substance of the hypothalamus. It increased gradually and became most abundant at the border of pars nervosa (Fig. 2). Neurosecretory cells in the hypothalamus, however, were not so remarkable in these stages. Twenty days after hatching the hypophysis was elongated and its posterior portion began to separate from the brain. One month after hatching, a typical pituitary stalk became noticeable at the anterior portion. In the hypophysis four divisions could now be recognized easily. But the cellular structure of each part was still different from that of the adult pituitary. More than a month was

Fig. 2. Hypophysis of two-month old goldfish. Neurosecretory substance (arrow) is recognized between the pars nervosa and pars intermedia. The other portion is a transitional region. GOMORI's CHP-staining. ×400

necessary till the gland attained the final structure as described by SCRUGGS ('51).

As is well-known the teleostean thyroid consists of a number of follicles scattered in tissues of the mandibular region. At the time of hatching, the gland developed as a solid mass of tissue at the dorsal region of the aorta, between the first and second gill slits. After 2 days, a few follicles became visible. They consisted of irregularly arranged epithelial cells and contained in the lumen a substance which could be stained faintly green with hematoxylin. In some individuals, however, the follicles were of the adult type, being composed of columnar epithelial cells and containing a strongly red-staining colloid with phloxin. The epithelium seem to be provided with a basal membrane. These follicles which contained the red-staining and green-staining colloid had never been observed to occur mixed in one individual. The number of fish having typical red-staining follicles increased gradually till it caught up that of fish with atypical green-staining follicles. But on the 10th day after hatching, the number of fish with the typical follicles decreased rapidly and almost all of individuals showed a faintly green-staining colloid. Staining reaction of the follicles changed again to red-staining in a few days, and 20 days after hatching all individuals had follicles with strongly red-staining colloid. After that no noteworthy changes were seen in the thyroid tissue throughout the experimental period.

Effect of hypophysectomy: In 24 cases out of 318, the hypophysectomy was successful. All of the specimens were fixed within a month after operation for the histological study.

At the time of operation, embryos had no melanophores in the entire body surface, except retinal pigment cells. A few hours after operation, melanophores began to differentiate at the anterior trunk region along the ventral ridge of myotomes as the cause of normal development, and soon extended posteriorly. Guanophores appeared in the eye in ten hours, and xanthophores also differentiated in the normal way.

In the control transparent fish the destruction of larval chromatophores began about 10 days after hatching and almost all of the pigment cells disappeared within a few weeks. Also in the

operated fish chromatophores degenerated in the corresponding stage in the normal way. The fish thus became completely transparent and albino, except the retinal pigment cells. It was apparent from the present data that hypophysis took no part in the manifestation of genetical specificity of the transparent type.

The specimens operated, however, showed various morphogenic disturbances. Some of them were malformed and incapable of development. Edemata of various degrees in the belly were commonly observed in early developmental stages. In later stages these larvae showed a characteristic dorsal bending of the trunk. In these cases the tip of the notochord grew anteriorly into the space of the hypophysis, reaching the floor of the parasphenoid (Fig. 3).

In several instances, regulation or regeneration of the epithelial hypophysis was observed. In these cases although the gland had regenerated as a whole, it was apparently abnormal in shape or smaller than that of the controls. Furthermore, fish having such

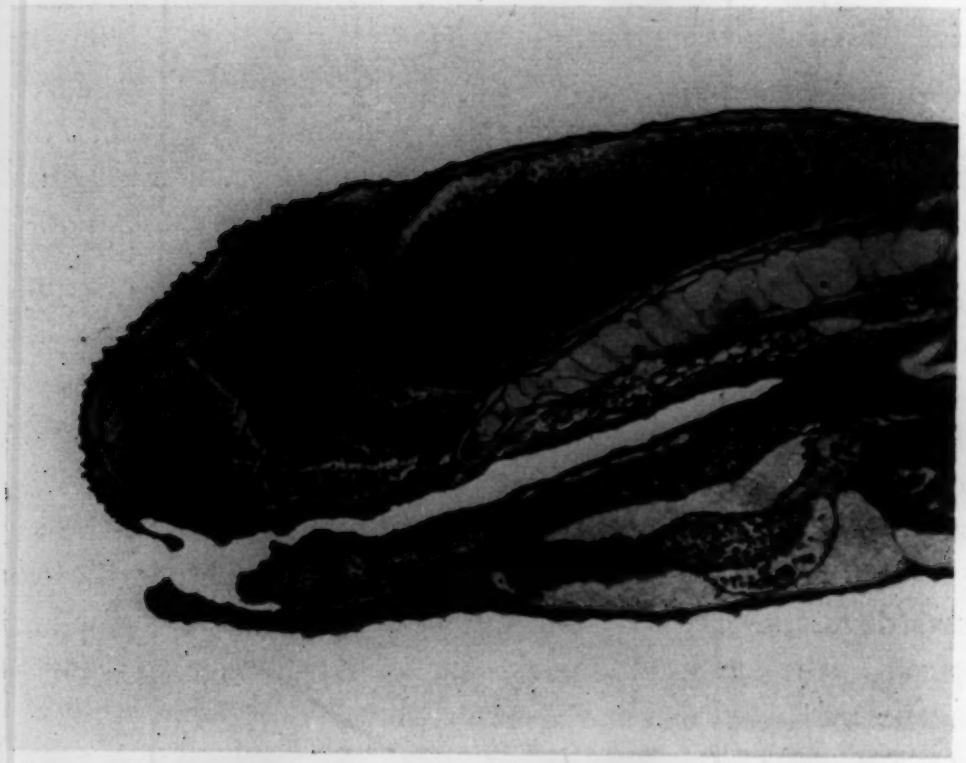

Fig. 3. Longitudinal section of the hypophysectomized larval goldfish. The tip of the notochord elongates to reach the parasphenoid bone. ×100

defective epithelial hypophysis underwent the chromatophor-degeneration.

The thyroid of the operated fish showed various states of development. In the oldest ones, fixed one month after operation, the gland consisted of follicles which were larger in size and less in number than the control. The epithelium was low and the follicles contained a deeply staining colloid. Judging from these histological criteria the thyroid gland was inactive. The thyrotropic function of the hypophysis was further indicated.

The effect of phenylthiourea: Retinal pigment granules began to differentiate within 40 hours after spawning in a control transparent fish, and the melanophores in the embryonic body became visible in 10 hours more. When treated with phenylthiourea, the differentiation of melanine granules was completely arrested both in the eye and in the body melanoblasts. The embryos remained perfectly transparent till the other two kinds of chromatophores differentiated. Guanophores appeared first in the iris and increased almost in the same sequence as in the controls, and the eye became refulgent. Guanophores were differentiated in the peritoneum and operculum also in the same way. Xanthophores ordinarily appered soon after hatching. In the experimental condition, they differentiated with a delay of more than five hours corresponding to the delay of hatching. About 2 weeks after hatching, the xanthophores and the guanophores began to degenerate, with some delay in comparison with control larvae. The treated specimens became again perfectly transparent and albino (Fig. 4).

Also in the normal fish, melanophores did not differentiated when treated with phenylthiourea. The fish showed a yellowish refulgence, because guanophores and xanthophores were formed in the normal way remained throughout the whole experimental period. The fish turned gradually from yellow to brownish yellow in colour (Fig. 5).

As mentioned above, the development of the treated specimens was retarded, and the proportion of the body became abnormal; *e.i.* the total body length became shorter whereas the depth and width became larger than normal. In most cases eyeballs were projected as in the telescope eyed goldfish.

Fig. 4

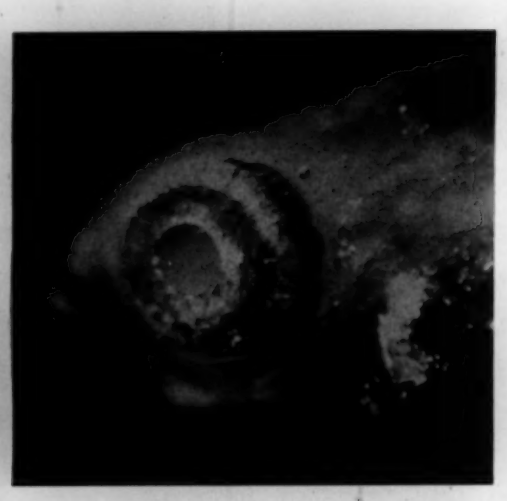

Fig. 5

Fig. 4 and 5. Two-month old goldfish, treated with phenylthiourea. Fig. 4. Transparent type (*TT*). Fig. 5. Normal type (*tt*). Guanophores of the eye and operculum have remained in normal type. *ca.* ×7

Fig. 6. Thyroid follicles (arrow) of the 2 month old goldfish, treated with phenylthiourea. The epitherial cells of the follicles become columnar, containing no colloidal substance. ×400

The histological observation showed a hyperplasia of the thyroid gland in the treated specimens. The follicles apparently increased in number, and contained small amount of colloid or were entirely

empty. The epithelial cell became slender and columnar (Fig. 6).

The hypophysis of the treated fish showed marked differences from that of the control. In many cases it was large and the vacuolized follicles were distinct in the center of the pars intermedia. But the neurosecretory substance in the pars nervosa showed no difference between treated and control animals.

Discussion

Since the early works of Swingle ('21), Allen ('30) and Atwell ('36), the intermediate lobe of the amphibian hypophysis has been considered to produce a melanocyte stimulating hormone (MSH). Since the same hormone is also believed to act on xanthophores, it is called the chromatotropic hormone (CTH). Recently, Bagnara ('58) has ascertained in anuran embryos that the guanophore contraction is also controlled by the same hormone. Thus the three kinds of chromatophores are under the influence of one pituitary hormone "CTH" in the amphibia.

On the other hand, Chavin ('56, '59) has ascertained that the intermediate hormone does not produce melanine granules in the xanthic goldfish, although in the gray goldfish, the hormone can disperse the granule in melanophores. In lipophores of the xanthic fish, he has also observed centripetal aggregation of the pigment granules and fast degeneration of the cells containing the aggregates following hypophysectomy. Thus the chromatophores of the young goldfish are affected by the pituitary hormone.

Fries ('43) has studied guanophores of *Fundulus* and *Cyprinodon*, according to whom, guanophores show a concentration or dispersion when the fish is kept on a black or white background respectively. However, since the response is abolished by means of ergotamine, which prevents melanophore dispersal, he has concluded that the hypophysectomy has no effect on the guanophore response in *Cyprinodon*.

In the present experiment, chromatophores of the larval goldfish do neither contract nor expand after removal of the pituitary primordium. In the fish of the transparent type the gene action responsible for the chromatophore-degeneration occurs as usual and the fish becomes completely transparent within a month despite of

hypophysectomy. It is apparent from this result that the hypophysis takes no part in controlling the gene action of transparent type.

In the normal development of the thyroid, the colloid of follicles shows an alternation of staining reaction about 10 days after hatching just before the chromatophore-degeneration. In some cases of hypophysectomy, the thyroid follicles look inactive while in the cases of phenylthiourea-treatment they are in a fully hyperfunctional condition. In both the cases there is no observable difference in the mode of the degeneration of larval chromatophores. Therefore the thyroid hormone too is considered to take no part in differentiation of the phenotype.

After feeding with the mammalian thyroid, TERAO ('22) has observed an acceleration of decolouration in young gray goldfish. In the wild-coloured goldfish BLACHER ('27) has found dispersion of melanophores and concentration of xanthophores after feeding large doses of thyroidin. The effect of the thyroid hormone upon the decolouration of the goldfish has also been demonstrated by the inhibition experiment with the antithyroid drug (HONMA and MURAKAWA, '57). The natural decolouration of young goldfish usually occurs in 2 to 3 months after hatching as the result of the degeneration of melanophores and xanthophores (KOKETSU, '15). Guanophores, however, remain unaffected. In the transparent type, as its racial characteristic, guanophores degenerate in an earlier stage than the natural decolouration occurs. Therefore, it may be assumed that the nature of the decolouration in the normal goldfish is entirely different from the chromatophore-degeneration of the transparent type.

SUMMARY

1. Influences of the pituitary and the thyroid gland on the chromatophore-degeneration in the goldfish of the transparent type have been studied, either by extirpation the pituitary primordium or by inhibiting the thyroid effect with phenylthiourea.

2. In the control fish, about 10 days after hatching and just at the beginning of the chromatophore-degeneration, it has been found that the neurosecretory substance becomes detectable in the pars nervosa, and the colloid of thyroid follicles changes its staining reaction.

3. It has been shown by hypophysectomy that the hypophysis takes no part in the manifestation of the genetical specificity of the transparent type.

4. By inhibiting the thyroid with phenylthiourea, it has been demonstrated that the chromatophore-degeneration in the fish of the transparent type is not under the control of the thyroid.

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(*Manuscript received: 31. Mar., 1960*)

EMBRYOLOGIA, Vol. 5, No. 2, pp. 139-169 (Juli, 1960)

UNTERSUCHUNG DES NUKLEINSÄURENGEHALTES IN VERSCHIEDENEN KEIMREGIONEN BEI DER FRÜHEN GASTRULA UND NEURULA VON *TRITURUS ALPESTRIS* (LAUR) UND *AMBYSTOMA MEXICANUM* (COPE)

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A. Einleitung

Eine große Zahl neuerer Untersuchungen weist auf die Bedeutung der Nukleinsäuren bei den während der Embryogenese ablaufenden Differenzierungsvorgängen hin (Brachet, 1947; 1951). Die Frage, ob die Nukleinsäuren auch bei der Induktion der Neuralplatte eine Rolle spielen könnten, versuchten verschiedene Autoren (Kuusi, 1951; Engländer und Johnen, 1957; Vahs, 1957) durch Versuche mit heterogenen Induktoren zu klären. Die Experimente konnten jedoch eine maßgebliche Beteiligung der Nukleinsäuren beim Induktionsvorgang nicht eindeutig nachweisen. Als Ausgangspunkt für weitere Untersuchungen erschien es von Bedeutung, die Verteilung der Ribonukleinsäure und Desoxyribonukleinsäure (RNS und DNS) in verschiedenen Keimregionen der Amphibiengastrula und -neurula festzustellen und etwaige quantitative Veränderungen während der Induktions- und Differenzierungsprozesse zu erfassen. Die erhaltenen Werte geben vielleicht eine Basis, auf der neuere Experimente zur Lösung der Frage nach der Natur der Induktionsstoffe aufbauen können.

Von besonderem Interesse sind unter diesem Gesichtspunkt a) das präsumptive Neuralmaterial der Gastrula, das noch keiner induktiven Beeinflussung ausgesetzt war; b) das gleiche Material nach erfolgter Induktion in der Neurula; c) der Organisatorbereich der Gastrula und d) das Urdarmdach der Neurula.

Für die Untersuchungen werden zwei Urodelenarten, *Triturus alpestris* (Laur) und *Ambystoma mexicanum* (Cope), herangezogen, da auf Grund der Arbeiten früherer Untersucher (Holtfreter, 1944; 1945; 1947; v. Woellwarth, 1952; Eyal-Giladi, 1954; Johnen, 1956) auf Unterschiede in der Reaktionsempfindlichkeit des Ektoderms geschlossen werden konnte.

Im Einzelnen sollen folgende Punkte untersucht werden:

1) Vergleich des RNS- und des DNS-Gehaltes verschiedener Keimregionen bei gleichem Alter.

2) Vergleich der RNS- sowie der DNS-Werte einer bestimmten Keimregion bei unterschiedlichem Alter.

3) Gegenüberstellung der RNS- und DNS-Werte gleicher Region und gleichen Alters bei *Triturus* und *Ambystoma*.

4) Vergleich zwischen *Triturus* und *Ambystoma* bezüglich des RNS- und DNS-Gehaltes verschiedener Regionen und dessen Verän-

derung im Verlauf der Entwicklung.*

B. Material und Methode

Das zur Untersuchung verwendete Material (Gewebestückchen von *ca.* 1.5 mm²) wurde jeweils der frühen Gastrula und Neurula von *Triturus alpestris* und *Ambystoma mexicanum* entnommen. Die Isolierung erfolgte zu dem Zeitpunkt, an dem die Keime den sichelförmigen Urmund zeigten (d.h. bei *Triturus* HARRISON-Stad. 10, bei *Ambystoma* LEAVITT-Stad. 10), oder wenn an den Keimen die Abzeichnung der Neuralplatte gerade begonnen hatte (d.h. bei *Triturus* HARRISON-Stad. 13, bei *Ambystoma* LEAVITT-Stad 13).

a. Nicht unterlagertes Ektoderm (*Triturus—Ambystoma*) (Abb. 1)

Der frühen Gastrula wurde *präsumptives Neural*-und *angrenzendes Epidermismaterial* entnommen und in der Mittellinie so zusammengeklappt, daß seine Innenseiten gegeneinanderlagen.

b. Unterlagertes Ektoderm (*Triturus—Ambystoma*) (Abb. 2)

Aus der frühen Neurula wurde die *craniale Hälfte* der sich gerade bildenden *Neuralplatte* entnommen und in der Medianen zusammengefaltet.

c. Obere Urmundlippe (*Triturus*) (Abb. 3)

Bei einem dritten Experiment wurde die *obere Urmundlippe* aus dem frühen Gastrulastadium isoliert.

d. Craniales Urdarmdach (*Triturus*) (Abb. 4)

Aus der frühen Neurula wurde das die craniale Hälfte der Neuralplatte unterlagernde Material, d.h. *Teile der prächordalen Platte, vorderer Chordabereich* und *angrenzende laterale Mesodermteile*, herauspräpariert.

---

* Herrn Priv.-Doz. Dr. Dr. H. ENGLÄNDER gebührt mein ganz besonderer Dank für die Anregung zu diesen Untersuchungen und für seine Unterstützung bei ihrer Durchführung.

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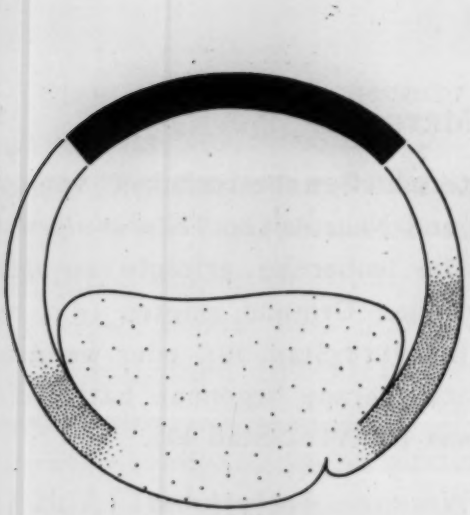

Abb. 1

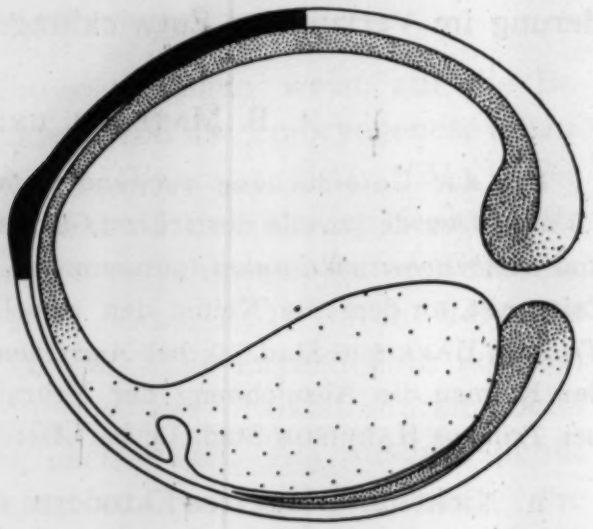

Abb. 2

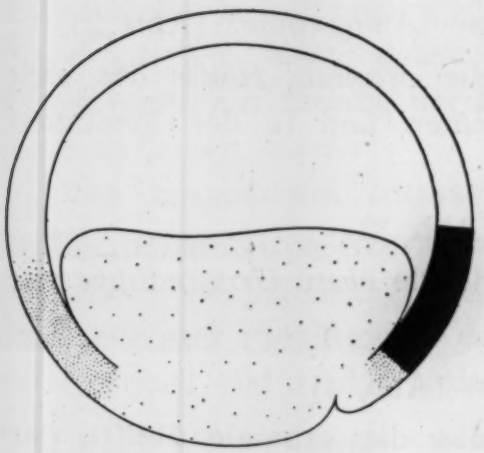

Abb. 3

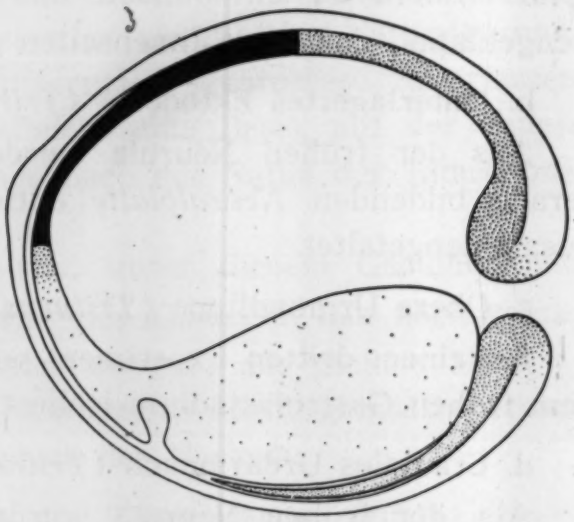

Abb. 4

Abb. 1–4. Operationsschemata
Isolierter Keimbezirk: schwarz.

Alle Isolate wurden entweder sofort nach der Operation extrahiert oder vor der Extraktion unter sterilen Bedingungen 5, 10 und 15 Tage in Holtfreter-Lösung mit einem Zusatz von 0.02% Debenalnatrium aufgezogen. Die Temperatur während dieser Zeit betrug 17–19°C. Die ersten drei bis vier Tage wurden die Explantate in mit Agar ausgegossenen Kulturschalen gehalten, um ein Anwachsen am Glasboden zu vermeiden, danach wurden sie in frisch sterilisierte Kulturschalen ohne Agar umgesetzt.

Um für die spätere Untersuchung meßbare Werte zu bekommen, wurden immer 30 Explantate gleichen Alters und der gleichen Region zu einer Serie zusammengefaßt und gemeinsam extrahiert.

Die chemische Extraktion läßt sich in zwei Abschnitte gliedern:

1) Die Entfernung der störenden säurelöslichen und lipoidartigen Bestandteile des Gewebes und

2) die Extraktion der Nukleinsäuren aus dem so vorbereiteten Gewebe.

In ihrem ersten Abschnitt erfolgte die Extraktion nach der von SCHNEIDER (1945) beschriebenen und später von KUUSI (unveröffentlicht) mitgeteilten Methode. Da bei diesem Verfahren aber RNS und DNS nicht voneinander getrennt werden, wurde die Extraktion weiter nach der Methode von OGUR und ROSEN (1950), die auch von STEINERT (1951), CHEN (1954) und ZELLER (1956) beschrieben ist, durchgeführt.

Folgendes Verfahren wurde der Bereitung der RNS- und DNS-Extrakte zugrunde gelegt:

Nach kurzer Alkoholfixierung und Homogenisierung der Explantate wurden die säurelöslichen Bestandteile durch Trichloressigsäure-Behandlung extrahiert, zur Entfernung der Lipoide wurde der Geweberest mit einem Alkohol-Äther-Gemisch erhitzt und nach Zentrifugierung die überstehende Flüssigkeit verworfen. Der noch verbleibende Geweberest trocknet im Vakuum innerhalb 15 min zu einem Pulver, dessen Gewicht genau bestimmt werden konnte und für die folgenden Photometermessungen eine feste Bezugszahl bot. Dieses Pulver bildete das Ausgangsmaterial für die Gewinnung des RNS- und DNS-Extraktes. Der Auszug der RNS erfolgte mit 5 ml 5 %iger Perchlorsäure über 20 h bei Zimmertemperatur. Nach dem Abzentrifugieren dieser Fraktion wurde der Geweberest mit 5 ml 5 %iger Perchlorsäure 40 min lang bei 70°C behandelt. Dabei ging die im Gewebe vorhandene DNS in Lösung, und nach dem Zentrifugieren blieb der DNS-haltige Extrakt zurück.

Die Extinktion dieser beiden Extrakte wurde im UV-Licht bei 230–290 mμ gegen reine Perchlorsäure als Leerwert bestimmt. Für die Messungen stand ein BECKMANN-Photometer zur Verfügung. Die erhaltenen Extinktionswerte konnten an Hand einer zuvor aufgestellten Eichkurve in Gammawerte umgerechnet werden, die dann auf 1 mg trockenes Gewebepulver bezogen wurden. Zu jeder Versuchsserie (d.h. 30 zusammen extrahierte Explantate) wurden Kontrollen durchgeführt. Sie wurden nicht extrahiert, sondern nach 10 bis 15 Tagen in Bouin fixiert, nach einer Stückfärbung in Boraxcarmin in Paraffin eingebettet, 8 μ dick geschnitten und dann mit Azan gegengefärbt.

Die in den folgenden Kapiteln gemachte Angabe über Alter der Explantate bezieht sich jeweils auf die Zeit zwischen Isolierung und Extrahierung.

Zur statistischen Auswertung wurden aus den einzelnen Gruppen die Mittelwerte und ihre zugehörigen mittleren Fehler berechnet, die im Text mit $\bar{y}$ und $s_{\bar{y}}$ angegeben sind. Die Differenz zwischen je zwei zu vergleichenden Mittelwerten wurde, falls der F-Test Homogenität der Varianzen erwiesen

hatte, mit dem *t*-Test auf ihre Signifikanz geprüft. Sofern die Versuchsanordnung es zuließ, wurde die mittlere Differenz auf Grund der Differenzen paarweise zugeordneter Werte getestet (Vergleich RNS-DNS). Sollten die Mittelwerte mehrerer Versuchsgruppen, die zu einem gemeinsamen Versuchskomplex gehören, untereinander verglichen werden, erfolgte die Prüfung der Differenzen auf Signifikanz mit dem Duncan-Test (studentisierte *t*-Verteilung), nachdem die Homogenität der Varianzen mit dem Bartlett-Test nachgewiesen worden war. Bei inhomogenen Varianzen wurde die Anzahl der Freiheitsgrade an Hand der relativen Anteile der zu vergleichenden Fehlerquadrate am Fehlerquadrat der Mittelwertsdifferenz ermittelt und bei der *t*-Prüfung in Rechnung gesetzt.

Die Differenzen sind im Text mit ihren ± Vertrauensgrenzen angegeben, die für 95 %ige Wahrscheinlichkeit berechnet sind. Sämtliche Einzelergebnisse, die bei der statistischen Auswertung berücksichtigt wurden, sind als Anhang der Arbeit angefügt (s.S. 166 ff.).

C. Beschreibung der Versuche

I. Feststellung des Nukleinsäurengehaltes isolierter Keimteile von Triturus alpestris (Laur)

a) Nicht unterlagertes Ektoderm

Es wurden drei Versuchsgruppen mit je 5 Serien zu je 30 Explantaten gebildet, wovon die Serien der ersten Gruppe sofort nach der Herausnahme extrahiert wurden. Die Serien der zweiten Gruppe wurden 5 Tage, die der dritten Gruppe 10 Tage kultiviert, bevor sie extrahiert wurden. Eine längere Kultur der Isolate war nicht möglich, da sie nach 10–12 Tagen zu zerfallen drohten. Bis zu diesem Zeitpunkt hatten sich die Explantate zu unregelmäßigen schwammartigen Gebilden entwickelt. Die histologische Untersuchung der Kontrollexplantate zeigte keine Differenzierungen; es hatte sich ausschließlich atypische Epidermis gebildet (Abb. 5).

Berechnet man für jede Gruppe den Mittelwert der *RNS-Messungen* und vergleicht diese Werte miteinander (Abb. 6, Anhang: Tabelle 1), so zeigt sich, daß in den 5 Tage alten Explantaten der RNS-Gehalt mit 95 %iger Sicherheit höher ist als in den Isolaten, die sofort nach der Entnahme extrahiert wurden. Die Differenz beträgt $3{,}0 \pm 1{,}18\ \gamma$/mg. Nach weiteren 5 Tagen—also bis zum 10. Kulturtag—hat der RNS-Gehalt um $1{,}76 \pm 1{,}37\ \gamma$/mg zugenommen.

Bei Berechnung der Mittelwerte der *DNS-Messergebnisse* (Abb. 6,

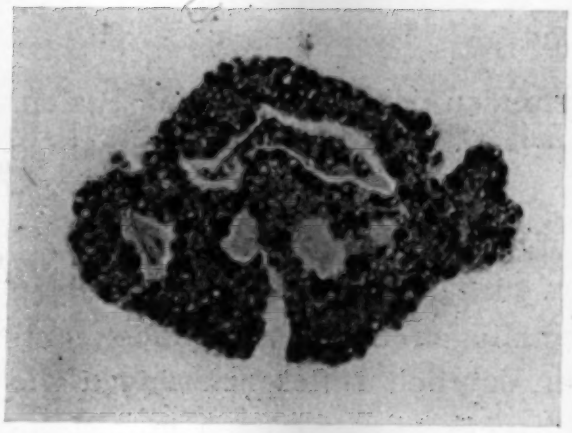

Abb. 5

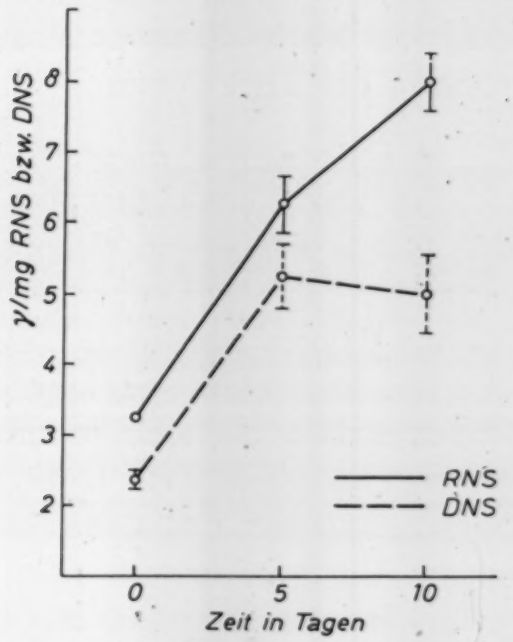

Abb. 6

Abb. 5. *Triturus alp.* Nicht unterlagertes Ektoderm. Schnitt durch ein 10 Tage altes Explantat: atypische Epidermis. Vergr.: 100 fach.

Abb. 6. *Triturus alp.* Nicht unterlagertes Ektoderm, HARR. 10, Mittelwerte der RNS- und DNS-Messungen mit ihren ± Abweichungen, $n = 5$. Abszisse: Kulturzeit in Tagen; Ordinate: RNS- bzw. DNS-Gehalt in γ/mg.

Anhang: Tabelle 2) läßt sich zwischen den 0 und 5 Tage alten Explantaten ein signifikanter Unterschied nachweisen. Er beträgt $2{,}86 \pm 1{,}3$ γ/mg. Die Differenz zwischen dem DNS-Gehalt der Isolate, die 5 und 10 Tage aufgezogen wurden, ist unter den vorliegenden Bedingungen nicht zu sichern, sodaß in diesem Zeitraum keine Änderung der DNS-Menge nachzuweisen ist.

Ein *Vergleich* der *RNS- und DNS-Werte* (Abb. 6, Anhang: Tabelle 1 u. 2) zeigt für alle Altersklassen des nicht unterlagerten Ektoderms einen höheren RNS- als DNS-Wert. Bei 0 und 5 Tagen beträgt die mittlere Differenz RNS minus DNS $0{,}96 \pm 0{,}65$ γ/mg. Am 10. Kulturtag ist dann die Differenz beträchtlich größer, da der DNS-Gehalt sich nicht mehr verändert, während der RNS-Wert zugenommen hat, sie beträgt jetzt $2{,}99 \pm 0{,}65$ γ/mg.

Diese *Ergebnisse* lassen den Schluß zu, daß sich in einem Zeitraum von 0 bis 5 Tagen RNS- und DNS-Gehalt, mit einem

bestimmten Abstand, parallel ändern. Nach dem 5. Tag weichen dann die Werte stark voneinander ab, verursacht durch stärkeren Anstieg des RNS-Wertes.

b) *Unterlagertes Ektoderm*

Die vier Versuchsgruppen bestanden jeweils aus fünf Serien. Die Isolate der ersten Gruppe wurden sofort nach der Entnahme extrahiert, die der zweiten wurden 5, die der dritten 10 und die der vierten Gruppe wurden 15 Tage aufgezogen und dann extrahiert.

Die Explantate wuchsen sehr gut, und schon nach wenigen Tagen hatten sie sich zu unregelmäßigen blasigen Gebilden entwickelt, an denen nach 10–12 Tagen deutlich Differenzierungen wie Augen, Balancer und Gehirnblasen zu erkennen waren. Die histologische Untersuchung der Kontrollexplantate ergab weitere Einzelheiten für den Charakter der differenzierten Zellkomplexe. In fast allen Fällen waren tel- und diencephale Bildungen vorhanden. Bei der Mehrzahl hatten sich Augen, zum Teil mit Linsen, gebildet. Nasen und mesencephale Strukturen fanden sich bei etwa der Hälfte der Fälle, während Strukturen des Met- und Myelencephalon nur ganz vereinzelt auftraten (Abb. 7).

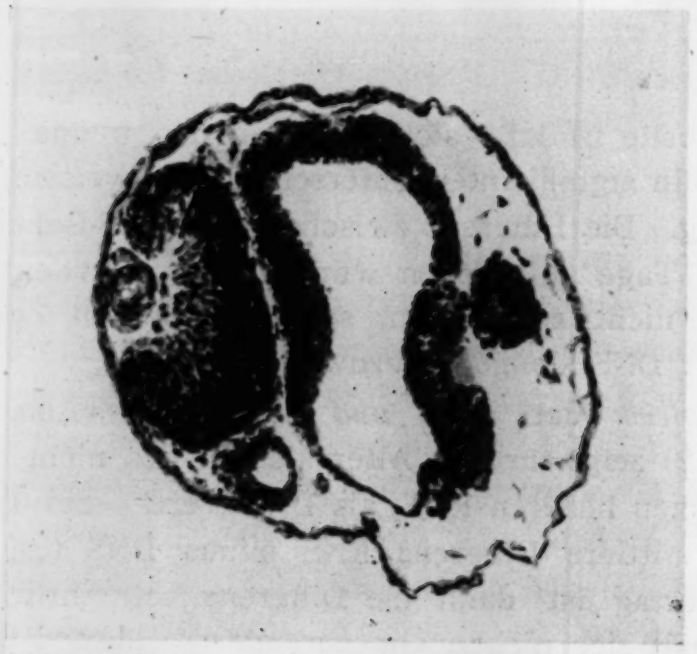

Abb. 7. *Triturus alp.* Unterlagertes Ektoderm. Schnitt durch ein 15 Tage altes Explantat: Auge mit Linse, Nase und Vorderhirnstrukturen Vergr.: 90 fach.

Auch bei den in diesem Stadium isolierten Keimteilen variieren die Mittelwerte der *RNS-Messungen* bei verschiedenem Alter eindeutig (Abb. 8, Anhang: Tabelle 1). In den ersten fünf Tagen läßt sich ein signifikanter Anstieg des RNS-Gehaltes von $6{,}43 \pm 1{,}0$ γ/mg feststellen. Bis zum 10. Kulturtag ist der RNS-Wert dann wieder abgesunken und zwar um $2{,}04 \pm 0{,}95$ γ/mg. Trotz dieses Rückgangs des RNS-Gehaltes ist der Wert von 10 Tagen noch durchschnittlich um 4,39 γ/mg größer als der von 0 Tagen. Zwischen dem 10. und 15. Tag ist die Menge der nachgewiesenen RNS wieder signifikant angestiegen und übertrifft zu diesem Zeitpunkt

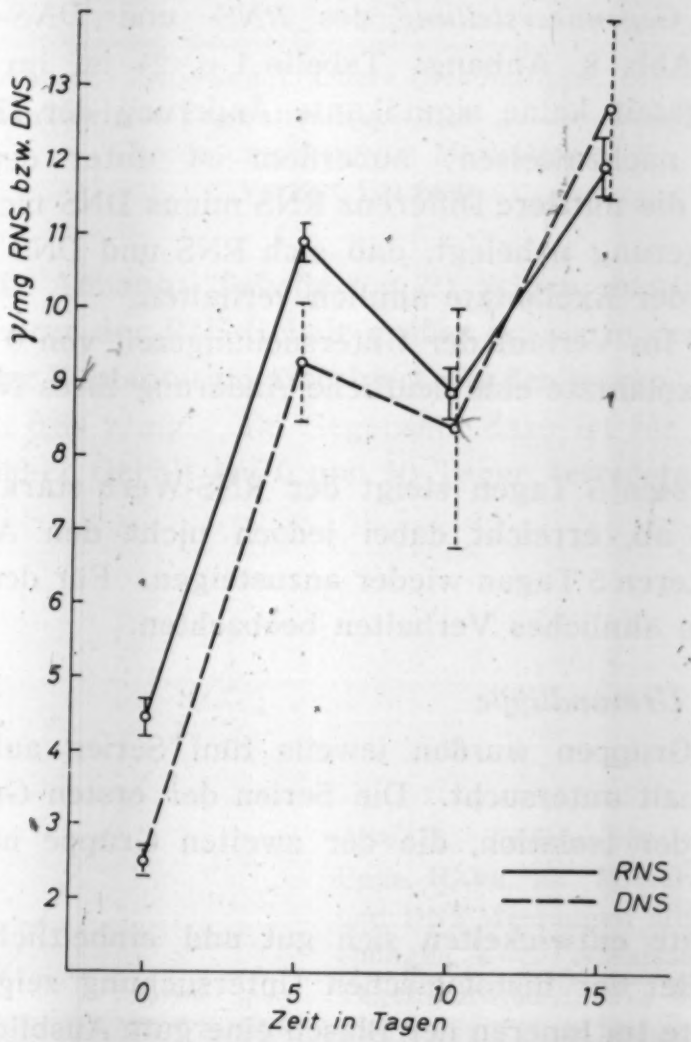

Abb. 8. ***Triturus alp.*** **Unterlagertes Ektoderm, HARR. 13. Mittelwerte der RNS- und DNS-Messungen mit ihren ± Abweichungen, $n = 5$. Abszisse: Kulturzeit in Tagen; Ordinate: RNS- bzw. DNS-Gehalt in γ/mg.**

den RNS-Gehalt der 5 Tage alten Explantate. Der Unterschied zwischen den Isolaten, die 10 und 15 Tage aufgezogen wurden, beträgt $3{,}11 \pm 1{,}0$ γ/mg, der zwischen 5 und 15 Tage alten Explantaten $1{,}07 \pm 1{,}0$ γ/mg.

Ein Vergleich des *DNS-Gehaltes* bei verschiedenem Alter (Abb. 8, Anhang: Tabelle 2) zeigt in der Zeit vom 0. bis zum 5. Tag einen signifikanten Anstieg von $6{,}76 \pm 3{,}05$ γ/mg. In den nächsten 5 Kulturtagen läßt sich eine Änderung des DNS-Gehaltes nicht mit Sicherheit feststellen; erst bei den 15 Tage alten Isolaten ist ein erneuter Anstieg des DNS-Wertes nachzuweisen, der gegenüber den 10 Tage alten Explantaten $4{,}33 \pm 3{,}49$ γ/mg beträgt.

Bei der *Gegenüberstellung* des *RNS-* und *DNS-Gehaltes* der Explantate (Abb. 8, Anhang: Tabelle 1 u. 2) ist im Verlauf der Untersuchungszeit keine signifikante Änderung der Differenz RNS minus DNS nachzuweisen; außerdem ist unter den gegebenen Bedingungen die mittlere Differenz RNS minus DNS nicht zu sichern, was die Folgerung nahelegt, daß sich RNS und DNS bei verschiedenem Alter der Explantate ähnlich verhalten.

Ergebnis: Im Verlauf der Untersuchungszeit von 0 bis 15 Tagen weisen die Explantate eine deutliche Änderung ihres Nukleinsäurengehaltes auf.

In den ersten 5 Tagen steigt der RNS-Wert stark an, sinkt bis zum 10. Tag ab, erreicht dabei jedoch nicht den Ausgangswert, um nach weiteren 5 Tagen wieder anzusteigen. Für den DNS-Gehalt kann man ein ähnliches Verhalten beobachten.

c) Obere Urmundlippe

In zwei Gruppen wurden jeweils fünf Serien auf ihren RNS- und DNS-Gehalt untersucht. Die Serien der ersten Gruppe wurden sofort nach der Isolation, die der zweiten Gruppe nach 10 Tagen extrahiert.

Die Isolate entwickelten sich gut und einheitlich zu blasigen Gebilden. Bei der histologischen Untersuchung zeigten alle Kontrollexplantate im Inneren der Blasen eine gute Ausbildung mesodermaler Strukturen wie Chorda- und Muskelzellen, während neurale Bildungen nur relativ selten und unvollkommen ausgebildet auftraten (Abb. 9).

Die *Mittelwerte* der *Nukleinsäurenmessungen* beider Altersgrup-

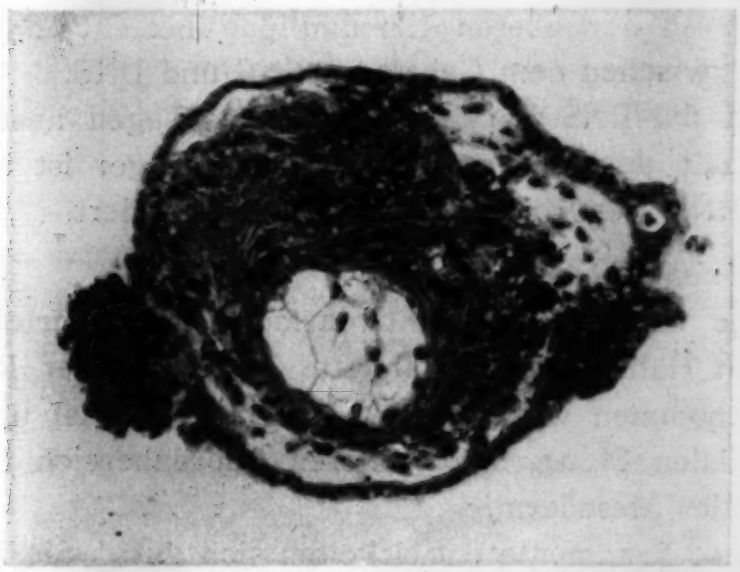

Abb. 9. *Triturus alp.* Obere Urmundlippe, Schnitt durch ein 10 Tage altes Explantat: gut ausgebildete Chorda umgeben von Muskulatur.
Vergr.: 100 fach.

pen (Abb. 10, Anhang: Tabelle 1 u. 2) zeigen deutlich, daß nach 10-tägiger Kultur der RNS-Gehalt größer ist als in den Isolaten, die sofort nach der Explantation extrahiert worden waren. Die Differenz beträgt $5{,}92 \pm 0{,}89\ \gamma$/mg. Im Gegensatz dazu ist für die DNS kein unterschiedlicher Gehalt bei 0 und 10 Tagen festzustellen.

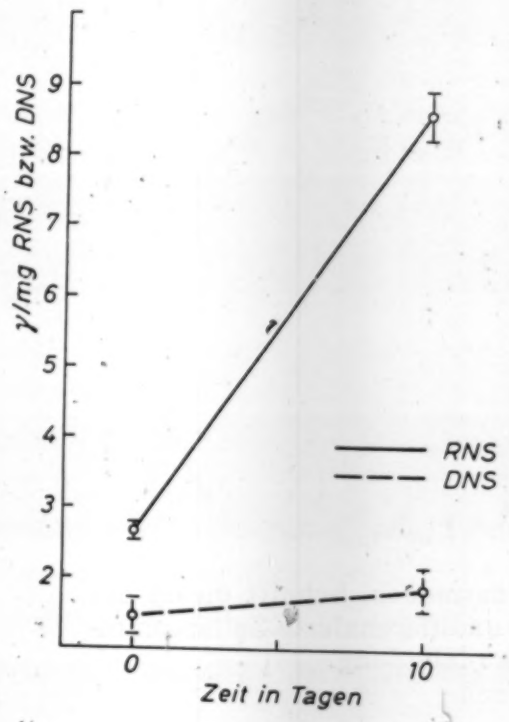

Abb. 10. *Triturus alp.* Obere Urmundlippe, HARR. 10. Mittelwerte der RNS- und DNS-Messungen mit ihren ± Abweichungen, $n = 5$. Abszisse: Kulturzeit in Tagen; Ordinate: RNS- bzw. DNS-Gehalt in γ/mg.

Ergebnis: In der oberen Urmundlippe besteht ein deutlicher Unterschied zwischen dem Gehalt an RNS und DNS.

Während der RNS-Wert von 0 nach 10 Tagen ansteigt, bleibt der DNS-Gehalt, der bei 0-tägiger Kultur geringer ist als der entsprechende RNS-Wert, bis zum 10. Tag unverändert.

d) Craniales Urdarmdach

Es wurde nur der Teil des Urdarmdaches verwertet, der unter der cranialen Hälfte der Neuralplatte lag, unter der Region also, die bei *b* entnommen wurde. Das isolierte Material umfaßt Teile der prächordalen Platte, den vorderen Chordabereich und angrenzendes laterales Mesoderm.

Auch diese Fragmente entwickelten sich gut. Sie ballten sich zuerst zu kleinen kompakten Kugeln zusammen, die dann im Verlauf von 15 Tagen ein mehr blasenförmiges Aussehen annahmen. Bei den histologisch untersuchten Kontrollexplantaten fiel die meist

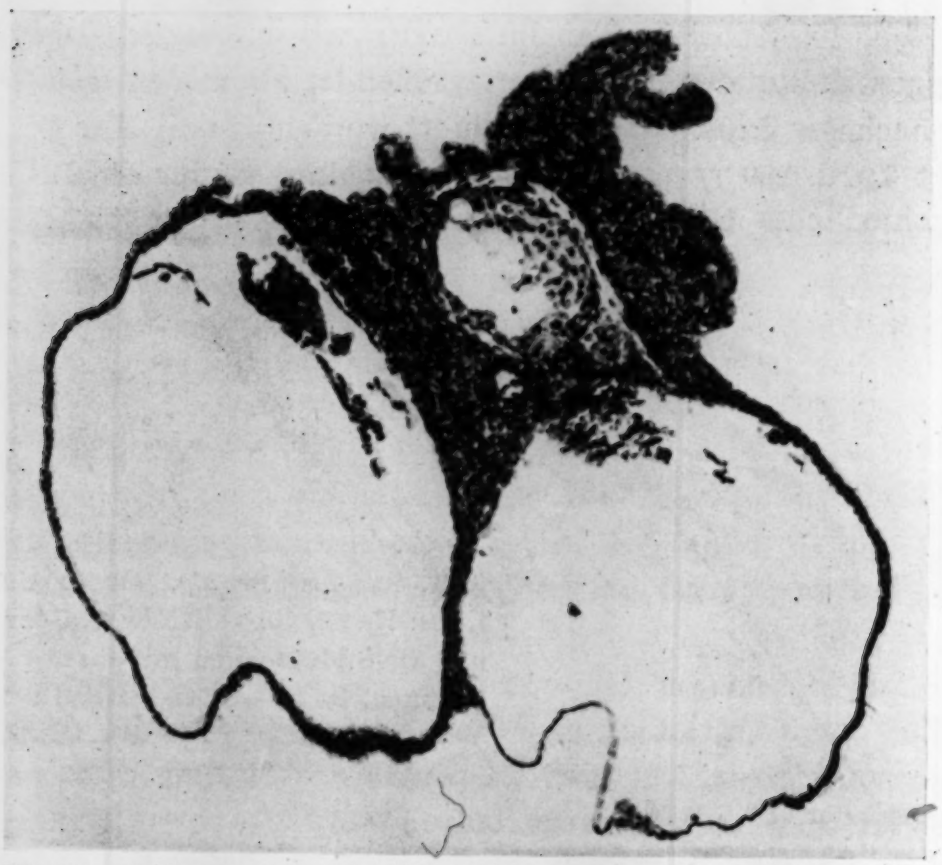

Abb. 11. *Triturus alp.* Craniales Urdarmdach, Schnitt durch ein 15 Tage altes Explantat: dotterhaltige undifferenzierte Zellansammlung, in der Mitte Chordabildung.
Vergr.: 70 fach.

sehr gut ausgebildete Chorda auf, die von undifferenzierten Zellansammlungen umgeben war (Abb. 11).

Der mittlere *RNS-Gehalt* des cranialen Urdarmdaches beträgt sofort nach der Explantation durchschnittlich 1,78 γ/mg. Zu diesem Zeitpunkt konnte eine mittlere *DNS-Menge* von 1,18 γ/mg festgestellt werden. Die demnach verhältnismäßig geringe Differenz zwischen RNS und DNS beträgt $0{,}6 \pm 0{,}23$ γ/mg (Anhang: Tabelle 1 u. 2).

Ergebnis: Im cranialen Urdarmdach ist der RNS-Gehalt größer als der DNS-Wert.

e) Vergleich der Nukleinsäurenwerte verschiedener Keimregionen gleichen Alters

Stellt man die *RNS-Werte* verschiedener Entwicklungsstadien und Keimregionen für eine Altersklasse zusammen, so ergibt sich folgendes Bild:

Bei RNS-Extraktion sofort im Anschluß an die Explantierung wird folgende Reihenfolge für steigenden RNS-Gehalt deutlich: Urdarmdach, obere Urmundlippe, nicht unterlagertes Ektoderm, unterlagertes Ektoderm, wobei alle vier RNS-Werte mit Sicherheit (95%) untereinander verschieden sind (Anhang: Tabelle 1).

Der RNS-Gehalt des unterlagerten Ektoderms ist bei 0 Tagen $1{,}22 \pm 0{,}76$ γ/mg höher als im 0 Tage alten nicht unterlagerten Ektoderm. Ebenso liegt der RNS-Gehalt im 5 Tage alten unterlagerten Ektoderm signifikant um $4{,}65 \pm 1{,}11$ γ/mg höher als im nicht unterlagerten Ektoderm gleichen Alters. Bei 10-tägiger Kultur ist zwischen den RNS-Werten des nicht unterlagerten Ektoderms und des unterlagerten Ektoderms und der oberen Urmundlippe kein gesicherter Unterschied festzustellen.

Bei einem Vergleich der *DNS-Werte* verschiedener Keimregionen sofort nach der Entnahme läßt sich zwischen dem unterlagerten und nicht unterlagerten Ektoderm kein Unterschied nachweisen, ebenso nicht zwischen oberer Urmundlippe und Urdarmdach (Anhang: Tabelle 2). Der DNS-Gehalt des unterlagerten Ektoderms ist mit 95 %iger Sicherheit größer als der der oberen Urmundlippe und der des Urdarmdaches. Der DNS-Gehalt des nicht unterlagerten Ektoderms ist dagegen signifikant größer als der des Urdarmdaches, nicht aber größer als der der oberen Urmundlippe. Bei 5-tägiger Kultur besteht ein signifikanter Unterschied zwischen nicht

unterlagertem und unterlagertem Ektoderm, das 4,04±2,13 γ/mg mehr DNS aufweist als das nicht unterlagerte. Am 10. Kulturtag zeigt das unterlagerte Ektoderm immer noch den signifikant höchsten DNS-Gehalt. Der Unterschied gegenüber dem nicht unterlagerten Ektoderm beträgt 3,34 ± 2,93 γ/mg und gegenüber der oberen Urmundlippe 6,47±3,34 γ/mg. Zwischen nicht unterlagertem Ektoderm und oberer Urmundlippe besteht zu diesem Zeitpunkt ebenfalls ein gesicherter Unterschied; in dem nicht unterlagerten Ektoderm lassen sich 3,13±1,34 γ/mg mehr DNS nachweisen als in der oberen Urmundlippe.

Ergebnis: Für die beiden untersuchten Nukleinsäuren läßt sich zusammenfassend feststellen, daß bei gleichaltrigen Isolaten die höchsten Werte immer im unterlagerten Ektoderm zu finden sind.

II. Feststellung des Nukleinsäurengehaltes isolierter Keimteile von Ambystoma mexicanum (Cope)

a) Nicht unterlagertes Ektoderm

In drei Versuchsgruppen wurden jeweils drei Serien zusammengefaßt, die aus je 30 Explantaten bestanden. Die Serien der ersten Gruppe wurden sofort nach der Entnahme extrahiert; die Serien der zweiten Gruppe wurden 5 Tage, die der dritten Gruppe 10 Tage kultiviert, bevor sie extrachiert wurden.

Die Isolate entwickelten sich ähnlich wie bei Triton zu unregelmäßigen Gebilden, die weder äußerlich noch bei histologischer Untersuchung Differenzierungsleistungen aufwiesen. Die Kontrollexplantate hatten ausschließlich atypische Epidermis gebildet.

Auch hierbei wurden die Mittelwerte der *RNS-Messungen* für jede Altersgruppe ermittelt und miteinander verglichen (Abb. 12, Anhang: Tabelle 3). In den ersten 5 Tagen der Kultur steigt der RNS-Wert mit Sicherheit an und zwar um 3,54±1,29 γ/mg. Ob nach weiteren 5 Tagen der RNS-Gehalt noch einmal zugenommen hat, kann nicht mit Sicherheit behauptet werden. Es läßt sich zwar eine mittlere Differenz von 1,13 γ/mg feststellen, die aber zu klein ist, um unter den vorliegenden Bedingungen als signifikant angesehen werden zu können.

Die Mittelwerte der *DNS-Messungen* zeigen in den einzelnen Altersgruppen ebenfalls deutliche Änderungen (Abb. 12, Anhang: Tabelle 4). Nach 5-tägiger Kultur finden sich in den Isolaten 4,32

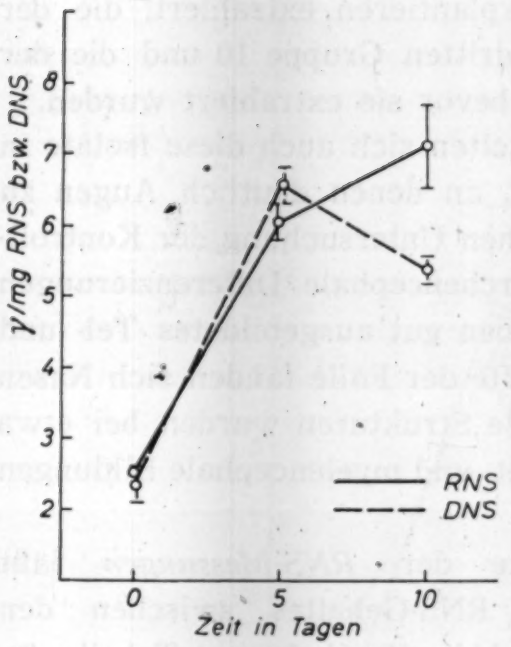

Abb. 12. *Ambystoma mex.* Nicht unterlagertes Ektoderm, LEAV. 10. Mittelwerte der RNS- und DNS-Messungen mit ihren ± Abweichungen, $n = 3$. Abszisse: Kulturzeit in Tagen; Ordinate: RNS- bzw. DNS-Gehalt in γ/mg.

±0,83 γ/mg mehr DNS als in den Explantaten, die sofort nach der Entnahme extrahiert worden waren. Bis zum 10. Kulturtag ist der DNS-Gehalt signifikant wieder abgesunken und zwar um 1,21 ± 0,8 γ/mg. Der Rückgang des DNS-Gehaltes ist aber nicht so groß, daß er den Ausgangspunkt—DNS-Wert der 0 Tage alten Isolate—erreichen würde. Die 10 Tage alten Explantate weisen immer noch einen signifikant höheren DNS-Gehalt auf als die sofort nach der Isolation extrahierten. Die Differenz beträgt 3,02 ± 0,8 γ/mg.

Ein *Vergleich* der *RNS- und DNS-Werte* (Abb. 12, Anhang: Tabelle 3 u. 4) ergibt bei 0 und 5 Tagen keinen sicheren Unterschied. Vom 5. zum 10. Kulturtag jedoch weichen der RNS- und DNS-Gehalt signifikant voneinander ab, und man kann zu diesem Zeitpunkt 1,83 ±0,75 γ/mg mehr RNS als DNS in den Explantaten nachweisen.

Ergebnis: In den ersten 5 Tagen zeigen die Werte für beide untersuchten Nukleinsäuren in übereinstimmender Weise einen Anstieg. Erst zwischen dem 5. und 10. Kulturtag wird ihr Verhalten verschieden: die RNS-Werte sind weiter angestiegen, während sich der DNS-Gehalt nicht verändert hat.

b) Unterlagertes Ektoderm

Drei Serien zu 30 Explantaten wurden zu einer Versuchsgruppe zusammengefaßt. Die Versuchsgruppen unterschieden sich untereinander durch verschiedenes Alter der Isolate. Die Serien der ersten

Gruppe wurden sofort nach dem Explantieren extrahiert, die der zweiten Gruppe wurden 5, die der dritten Gruppe 10 und die der vierten Gruppe 15 Tage aufgezogen, bevor sie extrahiert wurden.

Ähnlich wie bei *Triturus* entwickelten sich auch diese Isolate in wenigen Tagen zu blasigen Gebilden, an denen deutlich Augen zu erkennen waren. Bei der histologischen Untersuchung der Kontrollexplantate wurden in erster Linie archencephale Differenzierungen festgestellt. Die meisten Isolate ließen gut ausgebildetes Tel- und Diencephalon erkennen. Bei der Hälfte der Fälle fanden sich Nasen und Augen mit Linsen. Mesencephale Strukturen wurden bei etwa einem Drittel beobachtet, während met- und myelencephale Bildungen fast ganz fehlten.

Ein Vergleich der Mittelwerte der *RNS-Messungen* läßt charakteristische Änderungen des RNS-Gehaltes zwischen den einzelnen Altersgruppen erkennen (Abb. 13, Anhang: Tabelle 3). In den ersten 5 Kulturtagen nimmt der RNS-Wert des unterlagerten Ektoderms mit Sicherheit um $5{,}54 \pm 2{,}35$ γ/mg zu. Am 10. Tag lassen die Meßergebnisse ein Absinken des RNS-Gehaltes vermuten, was sich aber statistisch nicht belegen läßt, da die mittlere Differenz von 1,68 γ/mg im Rahmen der hier vorliegenden Bedingungen nicht als signifikant angesehen werden kann. In den folgenden Tagen—also bis zum 15. Tag—findet dann wieder ein Ansteigen des RNS-Gehaltes statt, das mit 95 %iger Sicherheit $3{,}22 \pm 2{,}35$ γ/mg beträgt. Nach 15 Tagen ist der RNS-Wert gegenüber dem der 0 Tage alten Explantate um $7{,}08 \pm 2{,}40$ γ/mg größer; gegenüber dem der 5 Tage alten Isolate besteht kein signifikanter Unterschied.

Der Mittelwert der *DNS-Messungen* (Abb. 13, Anhang: Tabelle 4) ändert sich in diesem Stadium signifikant nur zwischen 0 und 5 Tagen, und zwar nimmt er in diesem Zeitraum um $8{,}30 \pm 3{,}29$ γ/mg zu. Bis zum 10. Kulturtag findet dann keine Änderung des DNS-Gehaltes mehr statt; und auch der leichte Anstieg vom 10. zum 15. Tag, der im Mittel 2,51 γ/mg beträgt, ist unter den hier vorliegenden Bedingungen statistisch nicht zu sichern.

Stellt man in diesem Stadium die *RNS-* und *DNS-Werte* für die einzelnen Altersgruppen gegenüber (Abb. 13, Anhang: Tabelle 3 u. 4), so ist kein gesicherter Unterschied festzustellen. Man kann demnach annehmen, daß sich der RNS- und DNS-Gehalt bei zunehmendem Alter der Keimteile in paralleler Weise ändern.

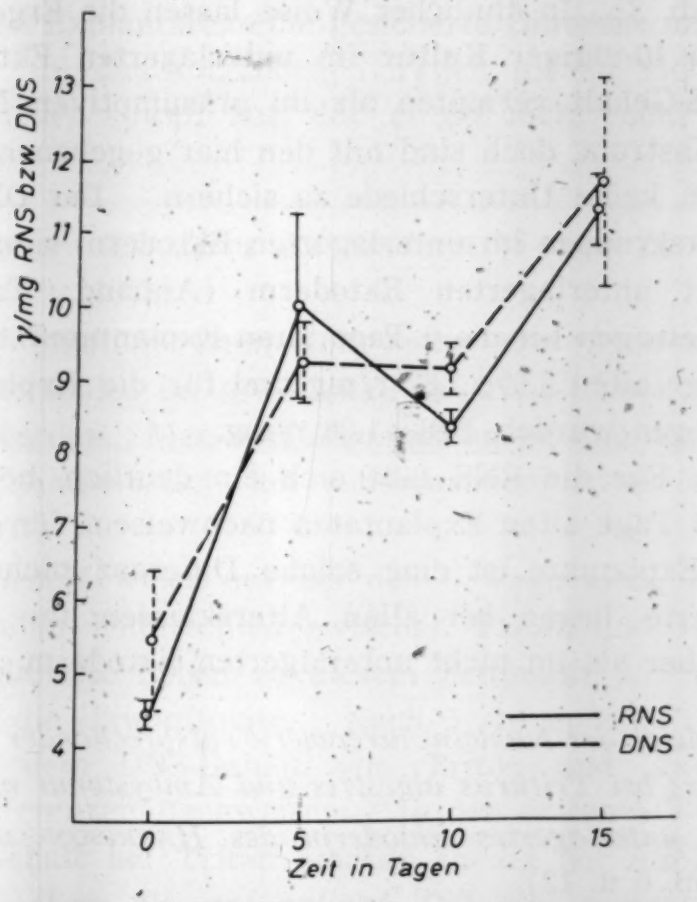

Abb. 13. *Ambystoma mex.* Unterlagertes Ektoderm, LEAV. 13. Mittelwerte der RNS- und DNS-Messungen mit ihren ± Abweichungen, $n = 3$. Abszisse: Kulturzeit in Tagen; Ordinate: RNS- bzw. DNS-Gehalt in γ/mg.

Ergebnis: Mit zunehmendem Alter kann man wie bei Triton eine gleichsinnige Änderung der RNS- und DNS-Werte feststellen. In den ersten 5 Tagen erfolgt ein starker Anstieg der Werte, an den sich bis zum 10. Tag ein geringes, statistisch nicht belegbares Absinken anschließt. Nach weiteren 5 Tagen hat der Nukleinsäurengehalt dann wieder deutlich zugenommen.

c) *Vergleich der Nukleinsäurenwerte des nicht unterlagerten und unterlagerten Ektoderms gleichen Alters*

Eine Gegenüberstellung der RNS-Werte des nicht unterlagerten und unterlagerten Ektoderms zeigt bei den Explantaten, die sofort nach der Entnahme extrahiert wurden, ein deutliches Überwiegen des RNS-Gehaltes um $1{,}97 \pm 0{,}15$ γ/mg im unterlagerten Ektoderm

(Anhang: Tab. 3). In ähnlicher Weise lassen die Ergebnisse bei 5- wie auch bei 10-tägiger Kultur im unterlagerten Ektoderm einen höheren RNS-Gehalt vermuten als im präsumptiven Neuralbereich der frühen Gastrula, doch sind mit den hier gegebenen statistischen Möglichkeiten keine Unterschiede zu sichern. Der DNS-Gehalt ist für alle Altersgruppen im unterlagerten Ektoderm signifikant höher als im nicht unterlagerten Ektoderm (Anhang: Tab. 4). Die Differenzen betragen für die 0 Tage alten Explantate $3{,}11 \pm 2{,}63\ \gamma$/mg, für die 5 Tage alten $2{,}69 \pm 1{,}82\ \gamma$/mg und für die Explantate, die 10 Tage aufgezogen wurden, $3{,}81 \pm 1{,}08\ \gamma$/mg.

Ergebnis: Für die RNS läßt sich ein deutlich höherer Gehalt nur in den 0 Tage alten Explantaten nachweisen, für die 5 und 10 Tage alten Explantate ist eine solche Differenz nicht zu sichern. Die DNS-Werte liegen bei allen Altersklassen im unterlagerten Ektoderm höher als im nicht unterlagerten Ektoderm.

III. Vergleich der Nukleinsäurenwerte entsprechender Keimregionen gleichen Alters bei Triturus alpestris und Ambystoma mexicanum

a) Nicht unterlagertes Ektoderm des HARRISON- *und* LEAVITT-*Stad. 10* (Abb. 6 u. 12).

Werden die Keimteile sofort nach ihrer Isolation extrahiert, besteht bezüglich des *RNS-Gehaltes* ein gesicherter Unterschied zwischen Triton- und Axolotl-Ektoderm. Der RNS-Wert ist bei Triton um $0{,}74 \pm 0{,}26\ \gamma$/mg größer als bei Axolotl. Nach 5 wie auch nach 10 Tagen ist eine Differenz des RNS-Gehaltes zwischen Triton und Axolotl nicht mit Sicherheit nachweisbar.

Man kann demnach feststellen, daß sich der anfängliche Unterschied im RNS-Gehalt nach 5 Tagen ausgeglichen hat, und daß für die folgenden Untersuchungszeiten die RNS-Werte bei Triton und Axolotl ein ähnliches Verhalten zeigen.

Ein Unterschied im *DNS-Gehalt* ist zwischen Triton und Axolotl im gleichen Stadium nur nach 5 Tagen festzustellen. Zu diesem Zeitpunkt weisen die Axolotlexplantate $1{,}34 \pm 0{,}95\ \gamma$/mg mehr DNS auf als die Tritonisolate. Sofort nach der Explantation und nach 10-tägiger Kultur lassen sich keine gesicherten Unterschiede im DNS-Gehalt bei Triton und Axolotl feststellen. Hier steigt also der DNS-Wert bei Axolotl von 0 nach 5 Tagen stärker an als bei Triton, so daß nach einem ähnlichen DNS-Gehalt sofort nach der Entnahme

bei 5 Tage alten Explantaten eine gesicherte Differenz nachgewiesen werden kann. In den folgenden 5 Tagen gleicht sich dann der Unterschied wieder soweit aus, daß er als nicht mehr signifikant gelten kann.

b) *Unterlagertes Ektoderm des Stadium 13 nach* HARRISON *und* LEAVITT (Abb. 8 u. 13).

In diesem Stadium kann für keine der Altersklassen ein gesicherter Unterschied im *RNS-Gehalt* zwischen Triton und Axolotl nachgewiesen werden. Man darf also annehmen, daß der RNS-Wert bei Triton und Axolotl sich mit zunehmendem Alter in ähnlicher Weise verändert.

Der *DNS-Gehalt* weist nur bei den 0 Tage alten Explantaten einen signifikanten Unterschied zwischen Triton und Axolotl auf. Die Axolotlexplantate haben zu diesem Zeitpunkt $2{,}77 \pm 2{,}23$ γ/mg mehr DNS als die Tritonisolate. Nach 5, 10 und 15 Tagen kann man zwischen dem DNS-Gehalt von Triton und Axolotl keine gesicherten Differenzen nachweisen. In den ersten 5 Tagen steigt also der DNS-Gehalt bei Triton stärker an als bei Axolotl, so daß sich bis zum 5. Tag die anfängliche Differenz ausgeglichen hat. Von diesem Zeitpunkt an ändert sich der DNS-Wert bei Triton und Axolotl in ganz entsprechender Weise.

Zusammenfassung der *Ergebnisse* von *Triturus* und *Ambystoma*:

Im Verlauf der Explantatentwicklung ist eine gute Übereinstimmung im Verhalten der Nukleinsäuren zwischen beiden Arten festzustellen. Nur in drei von insgesamt 14 vergleichbaren Wertpaaren kann eine Differenz gesichert werden.

D. DISKUSSION

Von den drei für die quantitative Nukleinsäurenbestimmung beschriebenen Methoden, die sich von den drei Bausteinen der Mononukleotide herleiten: Phosphorsäure, Ribose oder Desoxyribose und Purin- bzw. Pyrimidinbasen, wurde für die vorliegenden Untersuchungen ein Verfahren gewählt, das eine quantitative Bestimmung der Purin- und Pyrimidinbasen ermöglicht. Der Vorteil dieser Methode besteht darin, daß eine physikalische Eigenschaft der heterocyklischen Ringe, nämlich die Eigenschaft, das UV-Licht bei

260 mμ zu absorbieren, direkt—ohne Umweg über eine Farbreaktion—gemessen werden kann, was theoretisch eine äußerste Empfindlichkeit garantiert. Es muß allerdings ausdrücklich betont werden, daß die Versuchsergebnisse, die auf die oben beschriebene Weise gewonnen sind, nur relative Gültigkeit haben können, da die Bestimmungsmethode einige Fehlerquellen in sich birgt, die nicht ganz ausgeschaltet werden können und deshalb berücksichtigt werden müssen. Es handelt sich dabei um die von STEINERT (1951) aufgezeigte Tatsache, daß schon bei Zimmertemperatur ein gewisser Prozentsatz der Purine der DNS in Lösung gehen, wodurch die RNS-Werte etwas erhöht und die DNS-Werte etwas zu niedrig erscheinen. STEINERT versuchte diese Schwierigkeit zu umgehen, indem er die RNS-Extraktion mit konzentrierterer Perchlorsäure bei 0°C vornahm, wobei jedoch in 24 Stunden etwa 10% der RNS unhydrolisiert blieben. Da es in der vorliegenden Arbeit besonders auf den RNS-Gehalt ankommt, die DNS-Werte mehr eine Art Nebenbeobachtung darstellen, wurde die oben beschriebene Methode beibehalten. Weiterhin wurde die Methode von OGUR und ROSEN zur Nukleinsäurenbestimmung von SZE (1953) kritisiert, der die mit dieser Methode gefundenen DNS-Werte für zu hoch hält gegenüber den nach anderen Verfahren (SCHNEIDER 1945, SCHMIDT and THANNHAUSER 1945) gewonnenen Werten. Er vermutet eine oder mehrere Substanzen, vielleicht Proteinderivate, die mitextrahiert werden und das UV-Licht ebenfalls bei 260 mμ absorbieren. Auch CHEN (1954) weist auf diese Tatsache hin, sieht aber unter der Voraussetzung, daß Versuch und Kontrolle unter vollständig gleichen Bedingungen betrachtet werden, diesen technischen Fehler als vertretbar an. Die von ihm gefundenen RNS-Werte stimmen gut mit denen von STEINERT nach der gleichen Methode gewonnenen Daten überein.

Eine weitere Schwierigkeit ist die außerordentlich große und verschiedenartige Streuung der erhaltenen Werte, die zu einem Teil in der heterogenen Beschaffenheit und Herkunft des Materials selbst zu suchen ist. Die Versuche mit *Triturus* zum Beispiel erstreckten sich über zwei Laichzeiten, die Eier zu diesen Untersuchungen entstammen unkontrollierbar vielen Weibchen verschiedener Herkunft. Bei *Ambystoma* war das Versuchsmaterial einheitlicher, dementsprechend lag auch die Streuung innerhalb engerer Grenzen.

Unmittelbar vor bzw. nach der Invagination lassen sich die untersuchten Keimregionen von *Triturus* entsprechend der Zunahme des *RNS-Gehaltes* in folgender Reihenfolge anordnen: Urdarmdach, obere Urmundlippe, nicht unterlagertes Ektoderm und unterlagertes Ektoderm. Ähnliche Ergebnisse erbrachten die Experimente mit *Ambystoma*, die ebenfalls besagen, daß der RNS-Gehalt im neuralen Bereich der frühen Neurula höher ist als in der entsprechenden Region der frühen Gastrula.

Die *DNS-Werte* sind bei *Triturus* und *Ambystoma* weniger einheitlich. Während sich bei *Triturus* unterlagertes und nicht unterlagertes Ektoderm nicht voneinander unterscheiden, jedoch sicher höheren DNS-Gehalt aufweisen als obere Urmundlippe und Urdarmdach, die untereinander nicht differieren, zeigt bei *Ambystoma* das unterlagerte Ektoderm einen deutlich höheren DNS-Gehalt als das nicht unterlagerte Ektoderm.

Die 1953 von TAKATA veröffentlichten RNS-Werte verschiedener Regionen der frühen Gastrula und Neurula von *Triturus pyrrhogaster* bieten im engeren Rahmen das einzige Vergleichsmaterial für einzelne Werte der vorliegenden Untersuchungen, da die meisten in der Literatur zugänglichen Arbeiten RNS- und DNS-Werte ganzer Amphibieneier oder-embryonen angeben.

In Übereinstimmung mit TAKATA fanden wir, daß im Aktionsmaterial (obere Urmundlippe der frühen Gastrula und Urdarmdach der Neurula) signifikant weniger RNS enthalten ist als im Reaktionssystem (präsumptives Neuralmaterial der frühen Gastrula und induziertes Neuralmaterial der Neurula). TAKATA folgert aus seinen Befunden, daß im frühen Gastrula- wie im Neurulastadium die RNS entlang einem Gradienten verteilt ist, der seine Spitze im neuralen System hat. In ganz ähnlicher Weise beschreibt auch BRACHET (1947) auf Grund seiner histochemischen Untersuchungen einen animal-vegetativen, cephalo-caudalen wie auch dorso-ventralen Gradienten der RNS-Verteilung. Der Befund BRACHETS, daß die höchste RNS-Konzentration im Organisatorbereich zu finden sei, konnte weder durch die vorliegende Untersuchung noch durch die TAKATAS bestätigt werden.

Der von uns, jedoch nicht von TAKATA, beobachtete Rückgang des RNS-Gehaltes im Aktionsmaterial während der Invagination der

oberen Urmundlippe zum Urdarmdach, der in Einklang steht mit Ergebnissen BRACHETS und auch in den Untersuchungen von ROUNDS and FLICKINGER (1958) deutlich wird, ist wahrscheinlich im Zusammenhang zu sehen mit einer allgemeinen Intensivierung des Stoffwechsels in diesem Zeitraum, wobei die Reservestoffe des Dotters aufgebraucht werden, ihre Neubildung aber nicht so groß ist, den Verbrauch zu kompensieren. LØVTRUP (1955) konnte mit dem Einsetzen der Gastrulation einen Beginn der Proteinsynthese (siehe auch BRACHET, (1947) sowie ROUNDS and FLICKINGER (1958)), ferner einen Anstieg der Aktivität mehrerer Fermente feststellen, und er folgerte daraus, daß sich in dieser Phase ein "synthetisierender Mechanismus" ausbildet.

Ein Vergleich der RNS-Werte im nicht unterlagerten Ektoderm einerseits und unterlagerten Ektoderm andrerseits zeigt, daß vom Gastrula- zum Neurulastadium im Reaktionsmaterial ein deutlicher Anstieg sowohl des RNS -wie auch des DNS-Gehaltes erfolgt. Dieser Befund deckt sich mit den Daten, die von einigen anderen Autoren für den Gesamt-RNS- oder -DNS-Gehalt von Amphibienembryonen während der frühen Entwicklung veröffentlicht wurden. BRACHET (1947), KUTSKY (1950) und STEINERT (1951) berichten von einem mehr oder weniger klaren Ansteigen des RNS-Betrages mit der Gastrulation. Aus der gleichen Untersuchung, KUTSKY (1950), sowie aus Arbeiten von SZE (1953) und LØVTRUP (1955) geht hervor, daß zum gleichen Zeitpunkt auch mit einem Ansteigen des DNS-Gehaltes zu rechnen ist.

Diese Nukleinsäurenvermehrung muß unseres Erachtens in Zusammenhang gebracht werden mit dem erhöhten Zellstoffwechsel, der mit dem Induktionsprozeß und den anschließenden Differenzierungsvorgängen verbunden ist. Man kann annehmen, daß die für die Differenzierung notwendige RNS mit Beginn der Gastrulation im präsumptiven neuralen Bereich des Keimes neugebildet und angehäuft wird.

Eine Diffusion der RNS vom Urdarmdach in das darüber gelegene Ektoderm dürfte kaum wahrscheinlich sein, da sie gegen ein Konzentrationsgefälle erfolgen müßte, denn schon zu Beginn der Gastrulation findet sich im ektodermalen Bereich mehr RNS als in der Organisatorregion. Außerdem ist der Verlust an RNS von der

oberen Urmundlippe zum Urdarmdach geringer als die RNS-Zunahme im Ektoderm während der Invagination.

Im Gegensatz zu den vorliegenden Ergebnissen konnten ROUNDS and FLICKINGER (1958) ein deutliches Ansteigen des RNS-Gehaltes erst dann beobachten, wenn das neurale Ektoderm schon determiniert, d.h. die Neuralplatte schon angelegt ist, und diese Autoren folgern daraus, daß der Determinationsvorgang nicht an eine Erhöhung der RNS-Konzentration gebunden zu sein braucht. Wahrscheinlich liegt jedoch diese Diskrepanz der Ergebnisse weniger in einem Abweichen der Einzelwerte von den bisher berichteten begründet, als in einem völlig anderen Bezugssystem bei der Auswertung der Messungen. ROUNDS and FLICKINGER geben nämlich ihre RNS-Werte in RNS/DNS an, und es ist einleuchtend, daß bei annähernd gleichem Anstieg von RNS und DNS, wie wir es als wahrscheinlich annehmen, das Verhältnis RNS/DNS konstant bleibt. Eine Vermehrung der RNS tritt erst in Erscheinung, wenn der RNS-Gehalt stärker zunimmt als der der DNS. Ferner muß erwähnt werden, daß ROUNDS and FLICKINGER ihre Experimente an *Rana pipiens* durchführten; eventuelle Unterschiede in den Ergebnissen können also auf der Verschiedenheit des Versuchsmaterials beruhen.

Um die *Veränderungen des Nukleinsäurengehaltes* während der im Ektodermbereich nach erfolgter Induktion ablaufenden Differenzierungsprozesse zu verfolgen, wurden sowohl nicht unterlagerte als auch unterlagerte Ektodermteile verschieden lange Zeit als Isolate aufgezogen und die RNS- und DNS-Werte verglichen (Anhang: Tabelle 1, 2, 3, 4). Zunächst fällt innerhalb der einzelnen Stadien eine Übereinstimmung der RNS- und DNS-Werte bei entsprechendem Alter der Isolate auf. Ein Vergleich sowohl der an den beiden Tierarten erhaltenen Werte, wie auch der beiden Nukleinsäuren miteinander zeigt, daß sich die Meßergebnisse im allgemeinen decken. Nur in drei von insgesamt 14 vergleichbaren Wertpaaren ließ sich statistisch eine Differenz zwischen Triton und Axolotl sichern. Die gute Übereinstimmung bei den restlichen 11 Wertpaaren macht es fraglich, ob diesen Differenzen Wert beizumessen ist und ob sie nicht durch die schon aufgezeigten technischen Fehlerquellen verursacht sein können. Wir können annehmen, daß die

Isolate aus sich entsprechenden Keimregionen von Triton und Axolotl bei gleichem Alter in ihrem RNS- und DNS-Gehalt keine grundsätzlichen Verschiedenheiten aufweisen. Die RNS- und DNS-Werte entsprechender Regionen und Altersklassen decken sich also weitgehend, eine Tatsache, mit der nicht zu rechnen war, da man weiß, daß im Zellstoffwechsel RNS und DNS zwei vollständig verschiedene Plätze einnehmen und die Synthese dieser beiden Stoffe durchaus nicht gekoppelt zu sein braucht.

Bei den im frühen *Gastrulastadium* explantierten Keimteilen zeigt sich, daß von 0 bis 5 Tagen ein parallel verlaufender Anstieg des RNS- und DNS-Gehaltes erfolgt. Nach 10 Tagen weichen RNS- und DNS-Werte sicher voneinander ab, indem der RNS-Gehalt weiter zunimmt, wenn auch nicht mehr so stark wie bis zum 5. Tag, der DNS-Gehalt sich aber bei Triton nicht mehr signifikant ändert, bei Axolotl sogar wieder absinkt.

Bei den im *Neurulastadium* isolierten Keimteilen lassen sich bei Triton wie auch bei Axolotl keinerlei Differenzen zwischen den Daten für RNS und DNS statistisch nachweisen, so daß man hier mit einem vollständig gleichsinnigen Verhalten rechnen muß.

Interessante Zusammenhänge zeigen sich, wenn wir die hier wiedergegebenen Nukleinsäurenwerte in Beziehung setzen zu dem jeweiligen Differenzierungszustand der Explantate. Bei Triton sowie Axolotl wächst das isolierte *nicht unterlagerte Ektoderm* der Gastrula, praesumptiver Neuralplatten—und angrenzender Epidermisbereich, innerhalb der ersten 10 Tage auf die zwei- bis dreifache Größe, entwickelt aber nur undifferenzierte Epidermis und beginnt nach *ca.* 12 bis 13 Tagen zu zerfallen. Der RNS-Gehalt erfährt in diesem Zeitraum eine deutliche Steigerung, die in den ersten 5 Tagen erheblich größer ist als in den folgenden Tagen, wo der RNS-Anstieg sich wahrscheinlich einem Maximum nähert. Die RNS-Vermehrung dürfte mit der von STEINERT (1951), TAKATA (1953) u.a. erschlossenen Synthese zusammenhängen, die im normalen Keim bei der Gastrulation einsetzt und sich wahrscheinlich auch in den Explantaten noch kurze Zeit erhält.

Die Explantate aus *unterlagertem Ektoderm* von Triton und Axolotl wachsen in den folgenden 15 Tagen nicht nur sehr gut, sondern zeigen auch deutlich neurale Differenzierungsleistungen.

Gut ausgebildete Sinnesorgane und Gehirnteile finden sich in hoher Zahl, die Explantate sind viel länger lebensfähig als die dem Gastrulastadium entnommenen; in Einzelfällen kann man sie 20 Tage und länger kultivieren, ein Zeitraum, in dem die Differenzierung der induzierten Organe weiter fortschreitet. Es ist anzunehmen, daß die im Gastrulastadium beginnende RNS-Synthese sich auch nach den Explantationen im Stadium 13 in den isolierten Keimteilen fortsetzt, denn bis zum 5. Tag ist ein starker RNS-Anstieg in den Explantaten zu verzeichnen, der die RNS-Zunahme in den Isolaten, die im Stadium 10 entnommen wurden, weit übertrifft. Der in den folgenden 5 Tagen beobachtete Rückgang der RNS könnte bedingt sein durch die in diesem Alter stattfindenden besonders durchgreifenden Differenzierungsvorgänge und dem damit verbundenen erhöhten RNS-Umsatz, den die gleichbleibende Synthese nicht voll kompensieren kann. Haben in den 10 Tage alten Explantaten die determinierten Organanlagen einen gewissen Differenzierungsgrad erreicht, findet wahrscheinlich eine deutliche Entlastung des RNS-Zellstoffwechsels statt, die ein Ansteigen der Meßwerte in den 15 Tage alten Explantaten zur Folge hat.

Zusammenfassung

1) Verschiedene Keimregionen der frühen Gastrula und frühen Neurula von *Triturus alpestris* und *Ambystoma mexicanum* wurden isoliert und auf ihren RNS- und DNS-Gehalt untersucht. Dabei zeigte sich, daß der *RNS-Gehalt* bei Triton wie bei Axolotl im unterlagerten Ektoderm gegenüber allen anderen Keimteilen am höchsten ist. Die Regionen können mit fallender RNS-Konzentration in folgender Reihenfolge angeordnet werden: unterlagertes Ektoderm, nicht unterlagertes Ektoderm, obere Urmundlippe und Urdarmdach. Für den *DNS-Gehalt* kann bei Triton und Axolotl zwischen unterlagertem und nicht unterlagertem Ektoderm kein Unterschied gesichert werden. Ebenfalls läßt sich bei Triton keine Differenz zwischen dem DNS-Wert der oberen Urmundlippe und dem Urdarmdach nachweisen.

2) Die entnommenen Keimteile wurden jeweils als Isolat aufgezogen und die Veränderung der Nukleinsäurenwerte bei verschiedenem Alter untersucht.

a) Im nicht unterlagerten Ektoderm der *Triturus*- und *Ambystoma*-Keime steigt der RNS-Gehalt bis zum 10. Tag an, während der DNS-Wert nur in den ersten 5 Tagen zunimmt und sich danach bis zum 10. Tag nicht mehr nachweisbar ändert.

b) Der RNS-Gehalt des unterlagerten Ektoderms steigt bei *Triturus* und *Ambystoma* bis zum 5. Tag an, sinkt dann in den folgenden 5 Tagen wieder ab, um bis zum 15. Tag erneut anzusteigen. In der gleichen Weise nimmt der DNS-Wert bis zum 5. Tag zu, verändert sich dann aber bis zum 10. Tag nicht mehr. Nach 15 Tagen ist der DNS-Gehalt wieder angestiegen und unterscheidet sich nicht vom RNS-Wert gleichaltriger Explantate.

3) Eine Gegenüberstellung der RNS- und DNS-Werte zeigt sowohl bei *Triturus* als auch bei *Ambystoma* im nicht unterlagerten Ektoderm in den ersten 5 Tagen entsprechendes Verhalten, bis zum 10. Tag weichen dann die Werte signifikant voneinander ab, der RNS-Gehalt steigt weiter, während sich für den DNS-Wert keine Änderung mehr nachweisen läßt.

Für das unterlagerte Ektoderm sind bei *Triturus* sowie bei *Ambystoma* keine gesicherten Unterschiede zwischen dem Verhalten der RNS- und DNS-Werte während der Aufzucht als Isolat festzustellen.

4) Ein Vergleich zwischen *Triturus*- und *Ambystoma*-Keimteilen entsprechender Region und Alters zeigt für die beiden untersuchten Nukleinsäuren im allgemeinen ein gleichsinniges Verhalten.

Summary

1) The content of RNA and DNA was estimated quantitatively in different regions of early gastrulae and neurulae of *Triturus alpestris* and *Ambystoma mexicanum*. The results show that the concentration of RNA in *Triturus* as well as in *Ambystoma* is higher in the underlaid ectoderm than in all other regions. These can be arranged according to decreasing RNA contents: underlaid ectoderm, not-yet-underlaid ectoderm, dorsal blastopore lip, archenteron roof. Concerning the DNA concentration, no difference can be ascertained between underlaid and not underlaid ectoderm, and likewise there is no difference between the DNA amount of dorsal blastopore lip and cranial archenteron roof.

2) A part of the explants was cultivated in isolation for 5, 10 or 15 days to estimate the change of the nucleic acids contents during these periods and the following results were found:

a) Not underlaid ectoderm of *Triturus* and *Ambystoma* gastrulae: The RNA concentration increases during the time of cultivation (10 days), while the DNA value only rises during the first 5 days, and after that no changes occur.

b) Underlaid ectoderm of *Triturus* and *Ambystoma* neurulae: The RNA content rises till the 5th day of cultivation, decreases during the next 5 days and then increases again from the 10th to the 15th day. Similarly the DNA value rises during the first 5 days, but then does not change till the 10th day. For the following 5 days the DNA content increases again and after 15 days' cultivation the DNA concentration does not differ from the RNA value of the equally old explants.

3) Comparison of the contents of the two nucleic acids shows that in the not underlaid ectoderm of *Triturus* and *Ambystoma* the RNA and DNA values do not differ from each other till the 5th day. In the following 5 days the figures diverge very distinctly, because the RNA content continues to increase, while the DNA value does not change significantly.

During the whole time of cultivation no difference between RNA and DNA content could be found in underlaid ectoderm.

4) Comparison between *Triturus* and *Ambystoma* explants from homologous regions and of the same ages shows that there is no difference in behavior between RNA and DNA.

Tabellenanhang

Einzelwerte der RNS- und DNS-Messungen, sowie die Mittelwerte der einzelnen Altersgruppen und ihre zugehörigen mittleren Fehler.

Tabelle 1. *Triturus alpestris.* RNS-Werte in γ/mg

Untersuchte Keimregion		Alter der Explantate in Tagen 0	5	10	15
a) Nicht unterlagertes Ektoderm (HARRISON-St. 10)		3,21	6,6	7,25	
		3,22	5,52	9,16	
		3,13	6,12	8,9	
		3,27	5,32	7,23	
		3,4	7,66	7,5	
	$\bar{y}$	3,25	6,24	8,01	
	$s_{\bar{y}}$	0,04	0,42	0,42	
b) Unterlagertes Ektoderm (HARRISON-St. 13)		4,93	10,68	10,0	11,1
		4,89	11,24	8,17	13,21
		4,9	10,3	8,68	11,3
		3,77	11,6	9,2	11,7
		3,83	10,63	8,21	12,51
	$\bar{y}$	4,46	10,89	8,85	11,96
	$s_{\bar{y}}$	0,27	0,23	0,34	0,39
c) Obere Urmundlippe (HARRISON-St. 10)		2,8		7,69	
		2,58		7,82	
		3,0		9,22	
		2,41		9,05	
		2,6		9,23	
	$\bar{y}$	2,68		8,6	
	$s_{\bar{y}}$	0,1		0,33	
d) Urdarmdach (HARRISON-St. 13)		2,0			
		1,83			
		1,59			
		1,73			
		1,69			
	$\bar{y}$	1,78			
	$s_{\bar{y}}$	0,07			

Tabelle 2. *Triturus alpestris.* DNS-Werte in γ/mg

Untersuchte Keimregion		Alter der Explantate in Tagen 0	5	10	15
a) Nicht unterlagertes Ektoderm (HARRISON-St. 10)		2,45	4,28	4,4	
		2,38	4,83	5,58	
		2,32	6,34	5,53	
		2,64	4,38	3,38	
		2,06	6,33	6,22	
	$\bar{y}$	2,37	5,23	5,02	
	$s_{\bar{y}}$	0,09	0,46	0,51	
b) Unterlagertes Ektoderm (HARRISON-St. 13)		2,63	10,3	7,6	10,93
		2,57	11,1	12,37	15,3
		2,7	9,1	8,91	12,3
		2,94	6,38	5,25	9,36
		1,68	9,45	7,7	15,6
	$\bar{y}$	2,5	9,27	8,37	12,7
	$s_{\bar{y}}$	0,22	0,8	1,16	1,22
c) Obere Urmundlippe (HARRISON-St. 10)		1,73		3,0	
		1,19		1,52	
		0,75		1,77	
		2,18		1,77	
		1,52		1,4	
	$\bar{y}$	1,47		1,89	
	$s_{\bar{y}}$	0,24		0,29	
d) Urdarmdach (HARRISON-St. 13)		0,96			
		1,29			
		1,35			
		1,15			
		1,16			
	$\bar{y}$	1,18			
	$s_{\bar{y}}$	0,07			

Tabelle 3. *Ambystoma mexicanum.* RNS-Werte in γ/mg

Untersuchte Keimregion		Alter der Explantate in Tagen 0	5	10	15
a) Nicht unterlagertes Ektoderm (LEAVITT-St. 10)		2,31	5,8	8,12	
		2,73	5,86	7,36	
		2,48	6,5	6,08	
	$\bar{y}$	2,51	6,05	7,19	
	$s_{\bar{y}}$	0,12	0,22	0,6	
b) Unterlagertes Ektoderm (LEAVITT-St. 13)		4,05	10,22	8,38	11,8
		4,8	12,09	8,64	10,58
		4,6	7,76	8,0	12,3
	$\bar{y}$	4,48	10,02	8,34	11,56
	$s_{\bar{y}}$	0,22	1,25	0,19	0,51

Tabelle 4. *Ambystoma mexicanum.* DNS-Werte in γ/mg

Untersuchte Keimregion		Alter der Explantate in Tagen 0	5	10	15
a) Nicht unterlagertes Ektoderm (LEAVITT-St. 10)		2,32 2,78 1,92	6,53 6,15 7,03	5,6 5,0 5,47	
	$\bar{y}$	2,34	6,57	5,36	
	$s_{\bar{y}}$	0,25	0,26	0,18	
b) Unterlagertes Ektoderm (LEAVITT-St. 13)		5,84 6,8 3,72	9,71 10,0 8,06	9,15 9,79 8,58	14,4 9,95 10,7
	$\bar{y}$	5,45	9,26	9,17	11,68
	$s_{\bar{y}}$	0,92	0,6	0,35	1,38

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EMBRYOLOGIA, Vol. 5, No. 2, pp. 170–177 (July, 1960)

THE BEHAVIOR OF CAROTENOID AND DISTRIBUTION OF XANTHOPHORES DURING DEVELOPMENT OF THE MEDAKA (*ORYZIAS LATIPES*)

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Although a number of works have been reported on the origin and development of melanophores, comparatively little is known of xanthophores so far. OKA (1931) claimed that "coloured lipophores" in the medaka (*Oryzias latipes*) first appeared in the somite stage, but his finding was undoubtedly related to pigment cells other than xanthophores. The red chromatophores reported by BANCROFT (1912) in interspecies-hybrids of *Fundulus* may not also be xanthophores.

These misleading findings may be due to the insufficiency of the method of identification of this species of cells. Recently, HISHIDA and TOMITA (1957) found that the xanthophores of the medaka are stainable with Sudan III because they contain a large amount of fat granules. Using this method, they were also able to detect fat-containing cells distributed as the xanthophore pattern in the adult fish of the white variety.

As to the transference of carotenoid during development, an excellent study was made by STEVEN (1949) in the brown trout (*Salmo trutta*). According to him, carotenoids are transferred from the yolk of the egg to the body of the larva without apparent loss. This process takes place mainly in the later larval period.

Since living embryos of many teleostean fishes are transparent, affording excellent materials for direct observation of pigment cell behavior during their development, the author performed some experiments using the medaka in order to analyse the route of the carotenoid from the yolk to the xanthophores during development. The initial stage of the appearance and the distribution of xanthophores were also investigated.

Experiments and Observations

I. Larval xanthophores and the colouration of the dorsal part of embryonic body

Two homozygous strains of the medaka (*Oryzias latipes*), the orange-red and the white varieties, were used.[1] The white variety has no typical xanthophores, lacking carotenoid pigment entirely.

In the orange-red variety, yellow dots begin to appear on the dorsal surface of larvae two or three days after hatching. These dots are assumed to be larval xanthophores or "xanthoblasts", because of the fact that the dots do not appear at all in the white variety. When the larvae of the orange-red variety were put into concentrated sulphuric acid (98%), the dots suddenly became blue, indicating the presence of carotenoid. It was also shown that the colour of xanthophores of the adult fish is changed to blue in sulphuric acid.

In order to ascertain the effect of diet upon the colour of the yolk, spawning females were separately fed on various kinds of diet, and the eggs spawned after three days were examined. It was found that the colouration of the yolk depends upon the kinds of diet regardless of the genotype. Yellow eggs were obtained from the females fed on green algae plus tubificid worms, red eggs on red pepper plus tubificid worms and colourless eggs on tubificid worms alone.

The examination of xanthophores was performed on larvae two or three days after hatching, no food being given from hatching until examination. In the orange-red variety, there is a definite correlation between the intensity of the colouration of the yolk and the visibility of larval xanthophores. The more intense is the yolk colour, the easier is detection of the larval xanthophores. The larvae developed from colourless eggs have no coloured xanthophores at all. These facts suggest that the colour of larval xanthophores may have its origin in carotenoid derived from the yolk. In the white variety, on the contrary, the larvae developed from coloured

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[1] The white fish is usually female (AIDA 1921). In 1953, a white male appeared as a cross-over in the breed of Prof. YAMAMOTO. By mating this white male to white females, a white strain used in the present study was established.

eggs have no coloured xanthophores. This indicates that the lack of colouration of larval xanthophores depends upon the genotype.

In the orange-red variety, larval xanthophores appear just after hatching. Even if the embryo was stained with Sudan III as described later, no xanthophore was detected before hatching. However, it was observed that the dorsal part of the embryonic body developed from the deep coloured egg is diffusely coloured before hatching. Since there is a correlation between the diffuse colouration and the yolk colour, it may be due to carotenoid derived from the yolk. This colouration also changes to blue upon the sulphuric acid test (Fig. 1 *B*).

Fig. 1. *A*: Dorsal and head colour of embryo (Stage 30). *B*: Blue stains which appear in concentrated sulphuric acid (Stage 30).

II. Distribution of xanthophores

For the purpose of tracing the development of xanthophores from hatching to the adult stage, the fish were stained by immersing them into an alcohol solution of Sudan III for 15–20 minutes. The regions selected for examination were as follows: dorsal, ventral and lateral regions of the head; mid-trunk and hind-trunk; pectoral, dorsal, ventral, anal and caudal fins. In the respective regions, the number of xanthophores contained in an area of 0.15×0.15 mm^2 was counted. With regard to the adult fish, the number both on and under the scales was counted. It varies widely with the individual fish and the regions studied. Each number in Fig. 2 was obtained

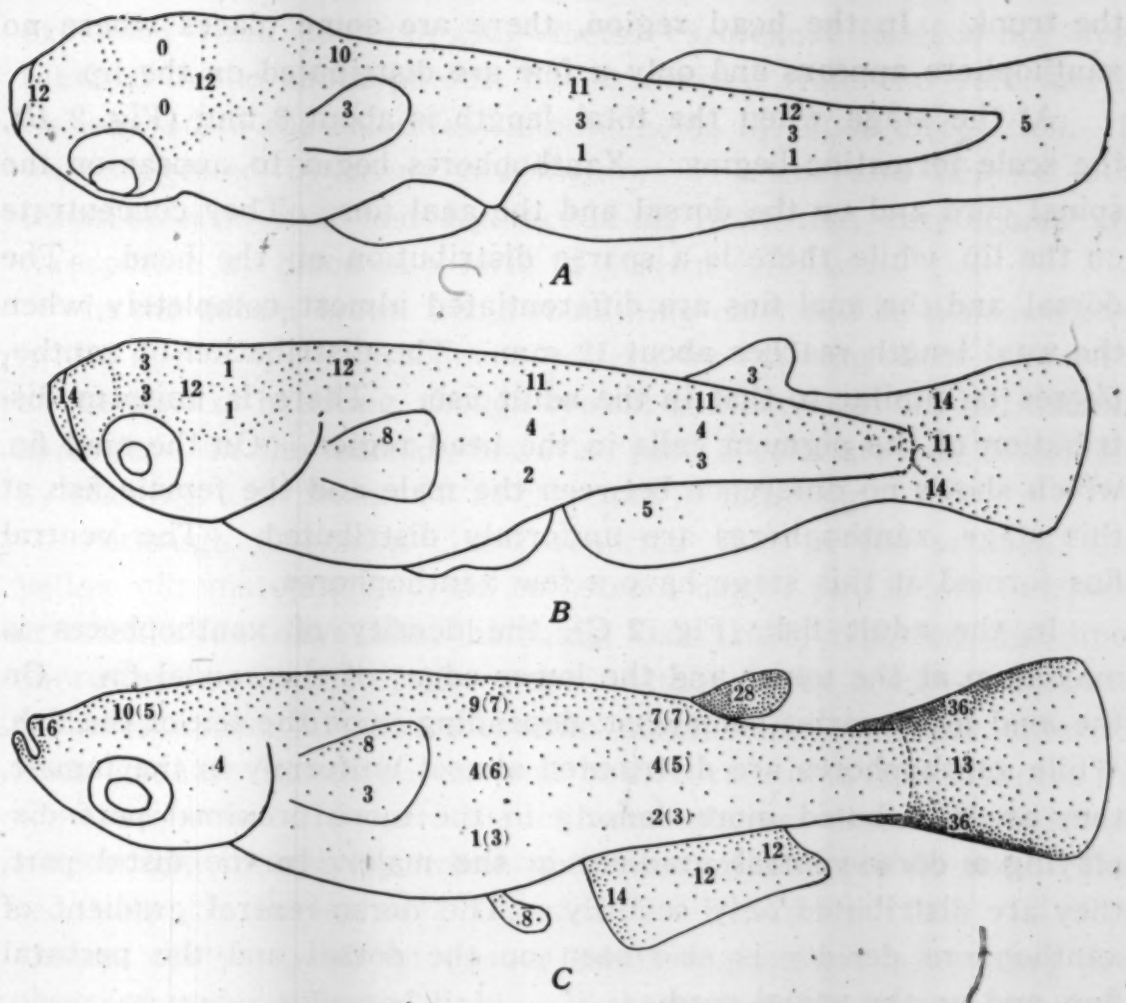

Fig. 2. Distributions of Sudan III stained xanthophores during development in orange-red variety. Numbers are those of xanthophores in 0.15×0.15 mm^2. Numbers out of parentheses in *C* show numbers of xanthophores on scales and the parenthesized numbers are those under scales. *A*: 5 mm long fry; *B*: 8 mm long fry; *C*: 33 mm long adult fish.

by averaging the data of 10 fish. The distribution of xanthophores along the spinal cord was also examined with the unstained material, as xanthophores could not be distinguished on a heavily stained spinal cord. The development after hatching was arbitrarily divided into four stages, according to the total length.

Just after complete absorption of the yolk, the total length of the larva reaches about 5 mm (Fig. 2 *A*). At this stage, the fins are not fully differentiated except for the pectoral and the caudal fins where xanthophores are distributed. A remarkable dorso-ventral gradient of the density of the xanthophore is found along

the trunk. In the head region, there are some places where no xanthophore appears and only a few are distributed on the lip.

At the stage when the total length is about 8 mm (Fig. 2 *B*), the scale formation begins. Xanthophores begin to appear on the spinal cord and on the dorsal and the anal fins. They concentrate on the lip, while there is a sparse distribution on the head. The dorsal and the anal fins are differentiated almost completely when the total length reaches about 12 mm. The distribution of xanthophores is similar to that in the adult fish. There is uniform distribution of the pigment cells in the head region. On the anal fin, which shows no difference between the male and the female fish at this stage, xanthophores are uniformly distributed. The ventral fins formed at this stage have a few xanthophores.

In the adult fish (Fig. 2 *C*), the density of xanthophores is maximum at the upper and the lower edges of the caudal fin. On the anal fin it varies somewhat, depending upon the sex of the fish. While xanthophores are distributed almost uniformly in the female, they are distributed more densely in the more proximal part displaying a dorso-ventral gradient in the male. In the distal part, they are distributed only scantily. The dorso-ventral gradient of xanthophore density is also seen on the dorsal and the pectoral fins, and on the spinal cord.

Discussion

Lowe (1917) reported in the trout (*Salvelinus pluvius*) that melanophores appear in the early embryonic stage, while xanthophores begin to appear two weeks after hatching. Steven (1949) also reported in the brown trout (*Salmo trutta*) that xanthophores are not usually apparent at the time of hatching, but the first pale yellow colouration are usually noticeable a few days after hatching on the tail, the dorsal and the future adipose fins. In the *Sillago sihama*, according to Ueno and Fujita (1954), both melanophores and xanthophores appear before hatching but the appearance of melanophores is much earlier than that of xanthophores. There is a similar tendency in the medaka that melanophores appear in the somite stage, whereas coloured xanthophores appear after hatching. The reason for the later appearance of xanthophores may be

explained from the following facts: carotenoid itself is not synthesized in the xanthophores, but is derived from the yolk and is transferred to the colourless xanthophores by blood circulation. It was suggested by many investigators (PALMER and ECKLES 1914, GANGULY *et al.* 1952 and KRINSKY *et al.* 1958) that carotenoids are transported by blood in a form of protein complex.

In the brown trout, lipoid-containing cells can be demonstrated in the fins as the xanthophore pattern by staining with Sudan IV at the time of hatching, though typical xanthophores are not usually apparent. In the medaka, however, such a cells are not detectable by staining with Sudan IV at the time of hatching.

Although GOODRICH (1927) reported that in the medaka, the yellow chromatophores could be noted a day or two before hatching or often a few days after hatching, OKA (1931) reported that lipophores appear in the somite stage. Although the brick-red chromatophores appear at the embryonic stage, they are quite different from genuine xanthophores and rather resemble guanophores in some respects. In fact, the chromatophores described by OKA reflect light intensively, while genuine xanthophores do not. The red yolk chromatophores reported by BANCROFT (1912) have a distinct contour with an opaque white appearance when they are observed under reflected light. It seems possible that these chromatophores are not xanthophores.

According to GOODRICH *et al.* (1941), carotenoid in the skin of the adult medaka is yellow lutein. Though his conclusion may be true under certain conditions, it does not generally apply at least for larvae. An evidence against his conclusion is that red carotenoid (capsanthin) can be introduced into eggs by feeding red pepper to the female, and is transferred to the dorsal part of embryos during development. Another evidence is that the fry reared on only red pepper from hatching to 10 mm long, have orange-red xanthophores and those reared on only dried spinach powder for the same period have yellow xanthophores.

SUMMARY

Larvae of the medaka developed from coloured eggs have coloured xanthophores, whereas those from colourless eggs have no coloured

xanthophores before feeding. Yolk carotenoid is transferred into the embryonic body and is accumulated in the dorsal part. The coloured xanthophores appear just after hatching and their distribution changes gradually during development. The reason for the late appearance of xanthophores in comparison with other chromatophores is discussed.

The author expresses his gratitude to Prof. T. YAMAMOTO under whose direction the present experiments were undertaken.

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CYCLIC CHANGES OF THE CORTEX AND THE CYTOPLASM OF THE FERTILIZED AND THE ACTIVATED SEA URCHIN EGG

II. THE FORMATION OF CLEAR SPOTS BY THE HYPERTONICITY, ETHER AND URETHANE

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KAWAMURA and DAN (1958) reported the cyclic appearance of SH-rich spots in etherized sea urchin eggs. On the other hand, the author preliminarily reported that when fertilized eggs and artificially activated eggs are exposed to urethane- or hypertonic sea water at a certain stage, clear spots appear in the cytoplasm, which behave also cyclically with the cleavage (KOJIMA, 1958). These findings are interesting from the viewpoint of the cyclic change of cytoplasmic condition. This paper is to report the results somewhat in detail.

MATERIAL AND METHODS

Materials used were eggs of *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*. In the first series of experiments, fertilized eggs were immersed into hypertonic sea water (8 cc. 2.5 N NaCl+ 50 cc. ordinary sea water) at intervals of 10 minutes starting from insemination. After respective 10 minute immersion, they were returned to ordinary sea water, and their cytoplasmic changes were watched continuously. Similar experiments were performed with eggs which had been activated artificially by means of butyric acid-sea water (6 cc. N/10 butyric acid+94 cc. ordinary sea water).

In the second series of experiments, fertilized eggs were stratified by centrifugation, and were treated with hypertonic sea water or urethane. In another series, unfertilized eggs were elongated or separated into two fragments by centrifugation at 10,000–15,000 *g* for 10–15 minutes which were activated artificially, and subjected to the same treatment.

Experimental Results

1. Effect of hypertonic treatment

A) The first series. Fertilized eggs were treated with hypertonic sea water for 10 minutes at various stages. When the treatment was applied at the prometaphase, clear spots of about 10 μ in diameter appeared near the egg periphery within 10 minutes after the eggs were returned to ordinary sea water. These spots gradually moved towards the early mitotic apparatus, increasing in size but decreasing in number, and finally, after about 15 minutes, disappeared. Then the amphiaster fully developed and cleavage followed.

In similar experiments with artificially activated eggs, clear spots were produced by the hypertonic treatment, only when the treatment was given at the early monaster-stage. The monaster developed after the spots had disappeared corresponding to the

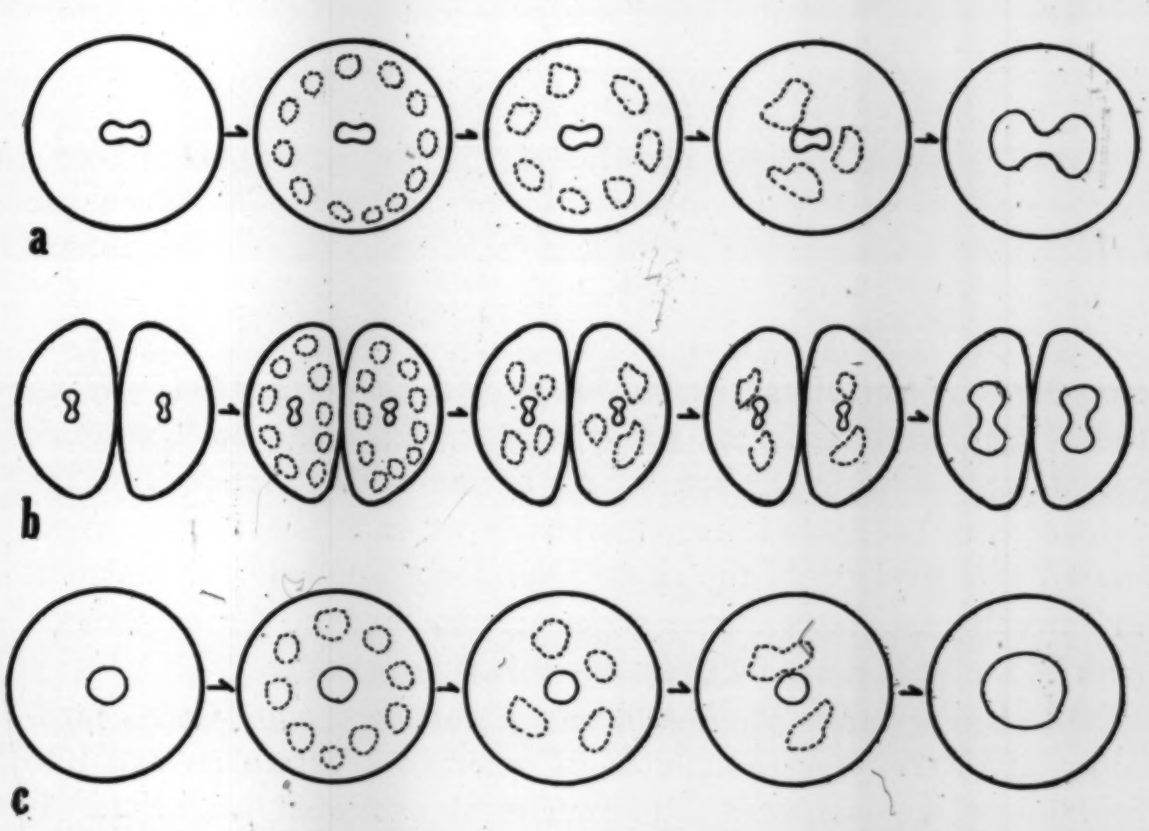
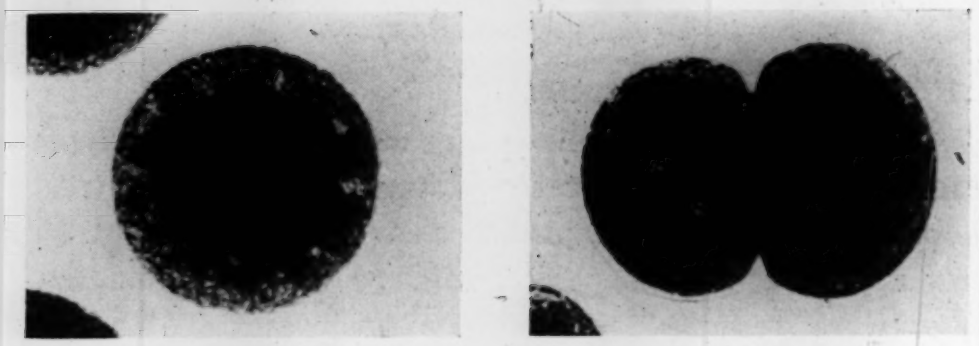


Fig. 1. Behavior of the clear cytoplasmic spots produced by hypertonic treatment in *Pseudocentrotus* eggs.——*a*: fertilized egg in 1-cell stage.——*b*: fertilized egg in 2-cell stage.——*c*: activated egg.



a *b*

Fig. 2. Fertilized *Pseudocentrotus* eggs treated with hypertonic sea water for 10 minutes at the prometaphase, showing the formation of spots in the cytoplasm.——*a*: the hypertonic treatment given at 65 minutes after fertilization; Photographed after 10 minutes in normal sea water.——*b*: similarly treated at 115 minutes after fertilization; Photographed at 135 minutes.

amphiaster of normally fertilized eggs.

The formation of the spots was observed until the third mitotic cycle both in the fertilized and in the activated egg. Figures 1–3 illustrate the spots in *Pseudocentrotus* eggs.

B) The second series. Fertilized eggs were centrifuged at 1,000 *g* until a clear zone were formed at the heavy pole. When the hypertonic treatment was applied to these eggs, clear spots tended to appear in a definite zone including the nucleus.

In the next group of experiments, unfertilized eggs were elongated by centrifugation at 10,000 *g* for 10–15 minutes. When these eggs were activated and then handled with the hypertonic sea water following the breakdown of the nuclear membrane, the formation of the spots in the eggs displayed various patterns according to the strength of the centrifugal force applied; for instance, in extreme case, most of the spots accumulated in the centrifugal part of the egg, quite far from the mitotic apparatus.

In another series of experiments, unfertilized eggs were centrifuged at 15,000 *g* for 15 minutes in order to separate an egg into centrifugal non-nucleated and centripetal nucleated fragments. It was found that even in an activated non-nucleated fragment, the formation of the spots was induced by the hypertonic treatment indicating that the formation of the spots is independent of the

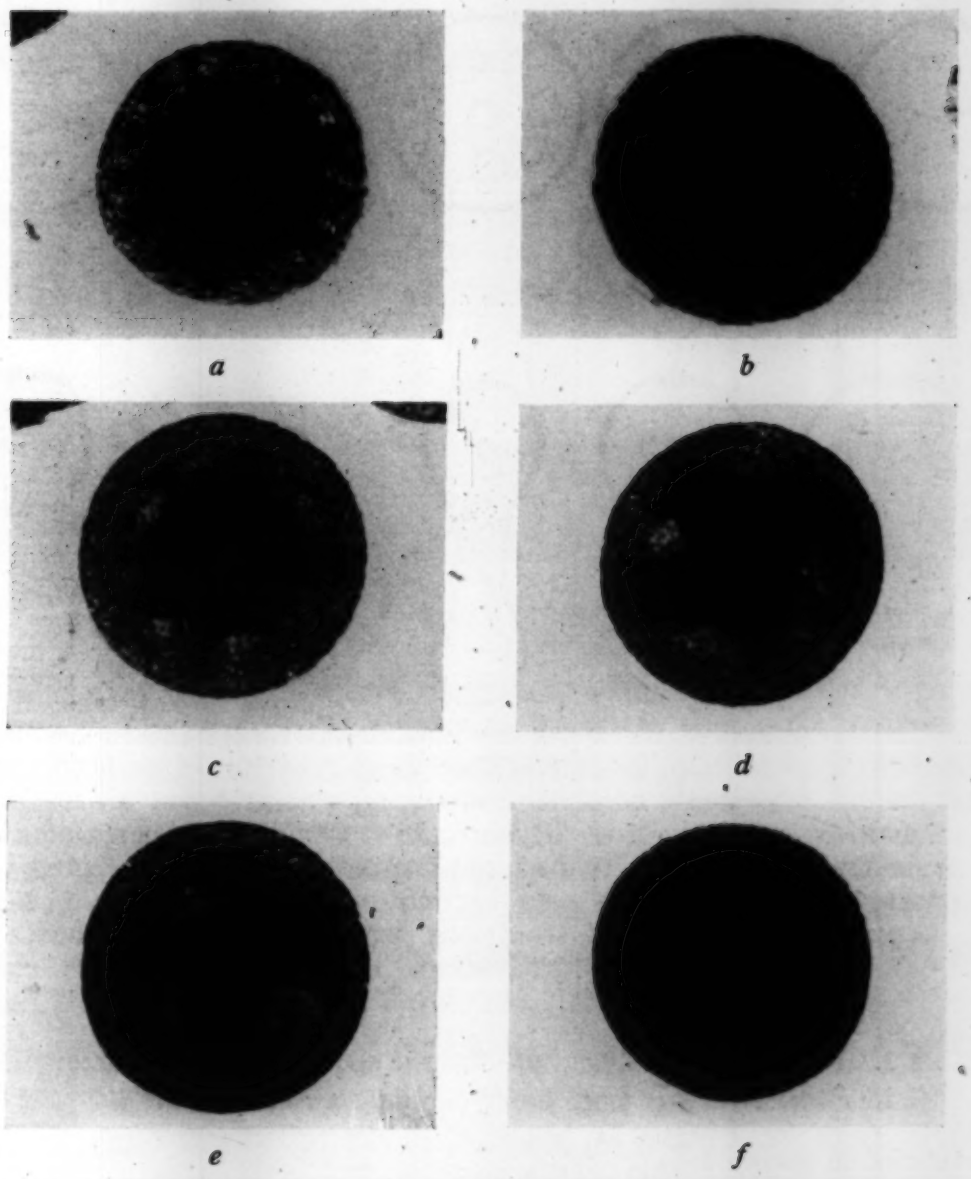


Fig. 3. Artificially activated *Pseudocentrotus* eggs, showing the appearance of the clear cytoplasmic spots by hypertonic treatment and their later disappearance (18°C.). Eggs were treated with hypertonic sea water for 10 minutes at the early monaster-stage (75 minutes after activation).

a: 10 minutes after the treatment.——*b–f*: their migration inward and coalescence and eventual disappearance.

nucleus. The formation of the spots in centrifuged *Hemicentrotus* eggs are shown in Figure 4–5.

C) Relation of the spot and the cytaster. Reference should be made here to the relation between the above-mentioned clear spots

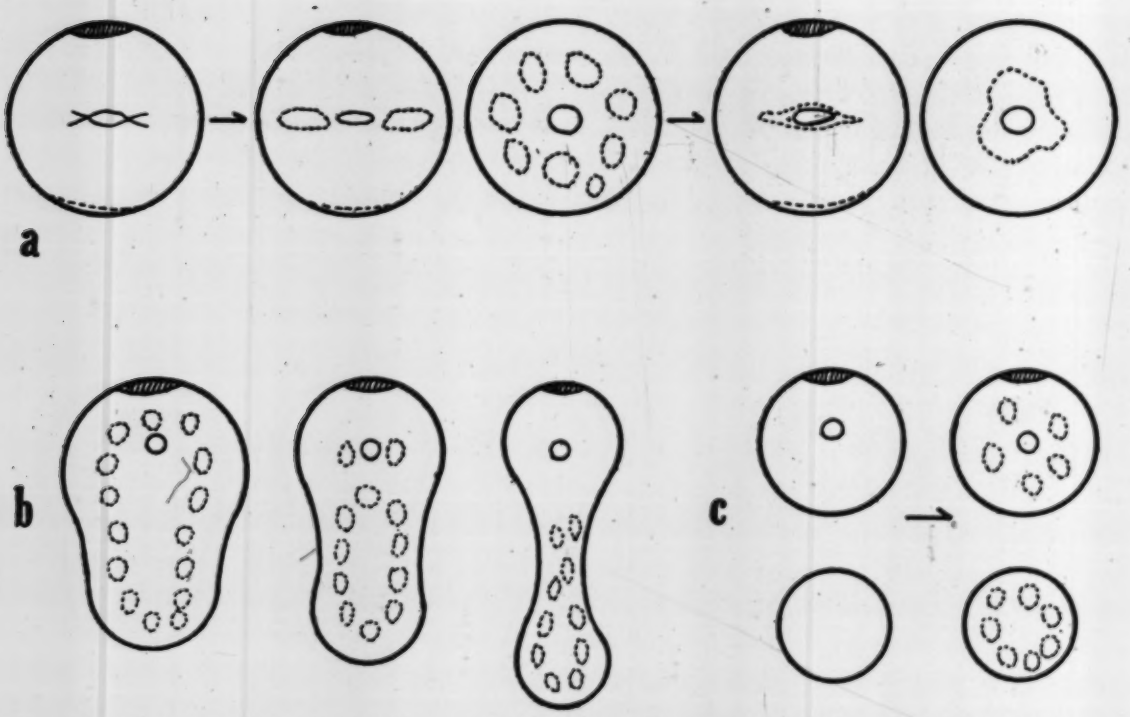


Fig. 4. The formation of the spots by hypertonic treatment in centrifuged *Hemicentrotus* eggs (20°C.).——*a*: fertilized eggs centrifuged at 1,000 *g* for 60 minutes before hypertonic treatment.——*b*: eggs centrifuged at 10,000 *g* for 10–15 minutes before activation. Hypertonic treatment was performed at the early monaster-stage.——*c*: activated egg-fragments: upper; nucleated, lower; non-nucleated. Centrifugation was carried out at 15,000 *g* for 15 minutes before activation. Hypertonic treatment was performed 60 minutes after activation.

Table 1. Formation of clear cytoplasmic spots (A) and cytasters (B) in *Pseudocentrotus* eggs by hypertonic treatment (20°C.).

Duration of hypertonic treatment (min.) ↓	Length of time between activation and hypertonic treatment (min.)→	15	25	35	45	55	65	75	85	95	105
10	A	−	−	−	−	−	±	+	±	−	−
	B	−	−	−	−	−	−	−	−	−	−
20	A	−	−	−	−	−	±	+	±	−	−
	B	−	−	−	−	−	−	−	−	−	−
30	A	−	−	−	−	−	±	+	±	−	−
	B	±	±	±	±	±	±	±	±	±	±
40	A	−	−	−	−	−	±	+	±	−	−
	B	+	+	+	+	+	+	+	+	+	+

A: records were taken at 10 minutes after the hypertonic treatment.
B: records were taken at 2 hours after the hypertonic treatment.
+: the formation of spots or cytasters in most of the eggs.
±: spots or cytasters only in some eggs.
−: no formation of spots or cytasters.

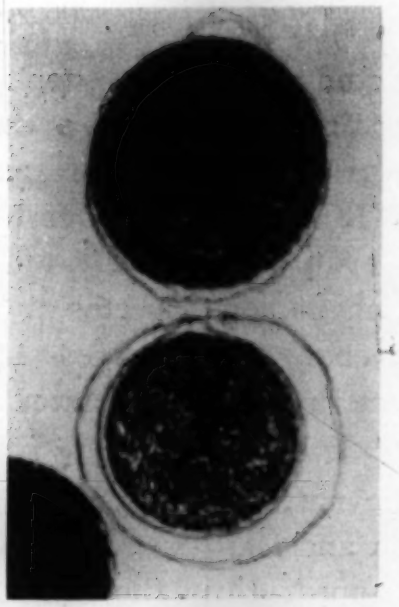


Fig. 5. Photograph of the spots in activated egg-fragments of *Hemicentrotus* (20°C.).

and the cytasters, both of which appear after the hypertonic treatment. It was found that a 10 minutes' hypertonic treatment is enough to induce the spots, while the treatment for 30 minutes or more is required to induce the cytasters. Beside this, the clear spots disappear when the eggs are returned to ordinary sea water, while the cytasters do not. These facts indicate some differences in the nature of the spots and the cytasters. An example in *Pseudocentrotus* eggs is shown in Table 1.

It should be added that when eggs are exposed to hypertonic sea water immediately after fertilization or activation, the cytasters do not appear until the prometaphase in fertilized eggs and the early monaster-stage in artificially activated eggs, respectively.

2. Effect of ether or urethane

KAWAMURA and DAN (1958) reported on the cyclic appearance of SH-rich spots in etherized eggs. In the present study, ether and urethane were used in order to compare the spot-forming effects of the two.

Eggs were exposed to a sea water solution of these reagents 10 minutes after fertilization and activation, respectively. It was found that the spots make their appearance cyclically in the cytoplasm both in the ether- (0.6%) and in the urethane- (0.06 M–0.08 M) sea water. The spots induced by using such reagents are quite similar in the mode of appearance to those induced by the hypertonic treatment.

Discussion

It has become clear from the present study that clear cytoplasmic spots can be produced by the hypertonic treatment at a certain stage of cleavage and this stage recurs prior to the aster formation both in fertilized and in activated eggs. It should not be overlooked that the formation of the spots occurs even in non-nucleated egg-fragments which are activated. This phenomenon seems to have something in common with the cyclic change in thickness of the hyaline layer which occurs also in activated non-nucleated egg-fragments (Kojima, 1960).

A question may be raised as to the nature and the origin of the clear cytoplasmic spots. Kawamura and Dan (1958) found that hyaline spots, abundant in SH groups, appear throughout the cytoplasm, if cleavage is suppressed with ether, which disappear soon after the eggs are returned to ordinary sea water. The astral growth occurs only after the disappearance of the SH spots. In the present paper a similarity is emphasized between the SH spots induced by ether and the clear spots produced by hypertonicity and urethane in their mode of appearance—their shape, their distribution and the stage in which they appear. All these facts suggest that that clear spots of the author may be formed as a result of the aggregation of SH-rich substance, which is generally considered to play an important in the aster formation (cf. Brachet, 1940; Mazia and Dan, 1952; Kawamura and Dan, 1958).

Summary

1) If fertilized eggs of the sea urchin are exposed to hypertonic sea water at the prometaphase and artificially activated eggs at the early monaster-stage, clear spots appear in the cytoplasm. Similar results are obtained when ether- or urethane-sea water is used instead of hypertonicity. The condition of the cytoplasm susceptible for forming the spots occurs cyclically in accordance with the cleavage cycle.

2) The same cyclic formation of the spots takes place even in activated non-nucleated egg-fragments.

3) It is suggested that these spots may be formed by the ag-

gregation of SH-rich substance which contributes to the aster formation.

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EMBRYOLOGIA Vol. 5, No. 2, 186-193 (July, 1960)

EXPERIMENTAL FORMATION OF THE MARK OF SPERM-ENTRY ON THE FERTILIZATION MEMBRANE IN THE SEA URCHIN EGG

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The author (1958 a and b) has preliminarily reported that when fertilized sea urchin eggs are transferred to an isotonic salt solution containing a large amount of Ca ions immediately after insemination, a ring-like structure appeares on the fertilization membrane at the point of sperm-penetration. The formation of such a structure is considered to have a close relationship to local change of the vitelline membrane at the time of fertilization. Some experiments have been performed in order to analyse the mechanism of this phenomenon.

MATERIAL AND METHODS

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were employed as material. In this species, separation of the fertilization membrane begins about 30 seconds after insemination and is completed within 35 seconds at 18°C. Ca-rich solution used for the formation of the sperm-entry mark was prepared by adding 10 ml of 10/27 M $CaCl_2$ to 90 ml of an isotonic salt solution (100 ml of 5/9 M NaCl plus 2.1 ml of 5/9 M KCl). The pH of the solution was adjusted to 8.2 by addition of 0.05 g of $NaHCO_3$ to 100 ml.

OBSERVATION AND EXPERIMENTAL RESULTS

1. Formation of the sperm-entry mark

Eggs were inseminated in usual way and, after 32 to 45 seconds, they were transferred to the Ca-rich solution. It was found that elevation of the fertilization membrane was stopped half-way, and in about 1 minute a cone-like protrusion appeared on the fertilization membrane at the point of sperm-penetration. The height of this cone soon reached a maximum, about seven microns, and then

gradually sank to the original level within 3 to 5 minutes. While the cone was flattening, a number of minute bodies were precipitated on the inner surface of the cone. After the cone became flat, more

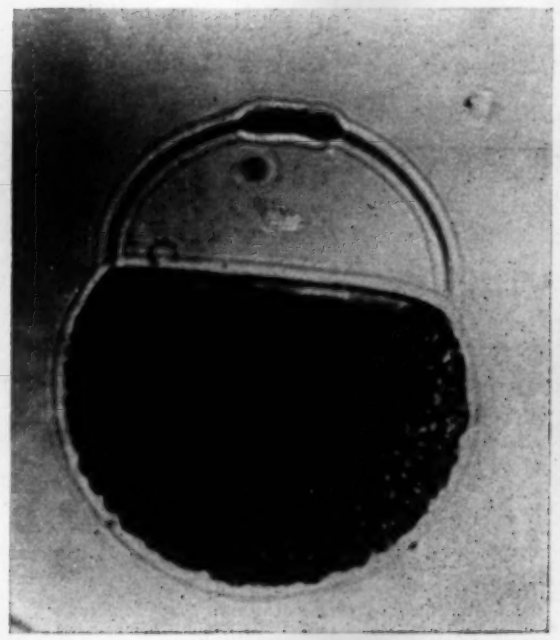

Fig. 1. Side view of a sperm-entry mark. ×*ca.* 400

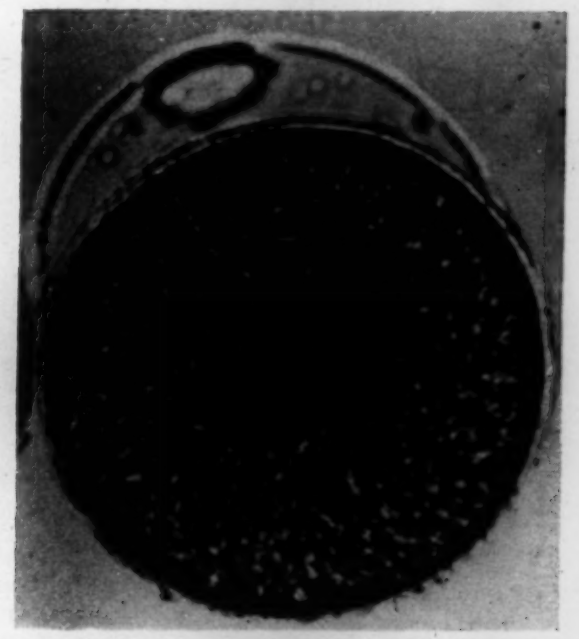

Fig. 2. A sperm-entry mark showing ring-like structure. ×*ca.* 500

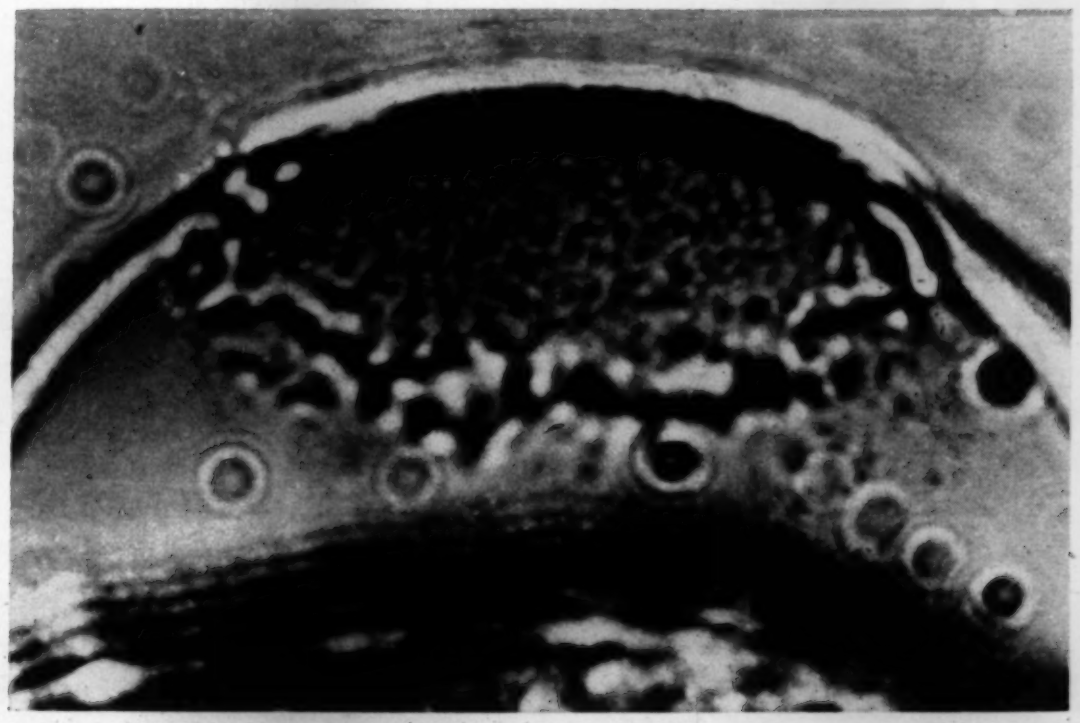

Fig. 3. A magnified sperm-entry mark showing granular membrane. ×*ca.* 5000

intense deposition took place around the base of the cone, forming a tiny ring-like structure (Figs. 1 and 2). In the previous paper (1958), this ring was designated as "sperm-entry mark".

The membrane inside of the ring exhibited a granular appearance while the membrane outside was smooth (Fig. 3). The ring was 5 to 20 microns in diameter, the commonest size being 10 microns. In a dispermic egg produced by heavy insemination, 2 marks corresponding to 2 spermatozoa were formed as shown in Fig. 4.

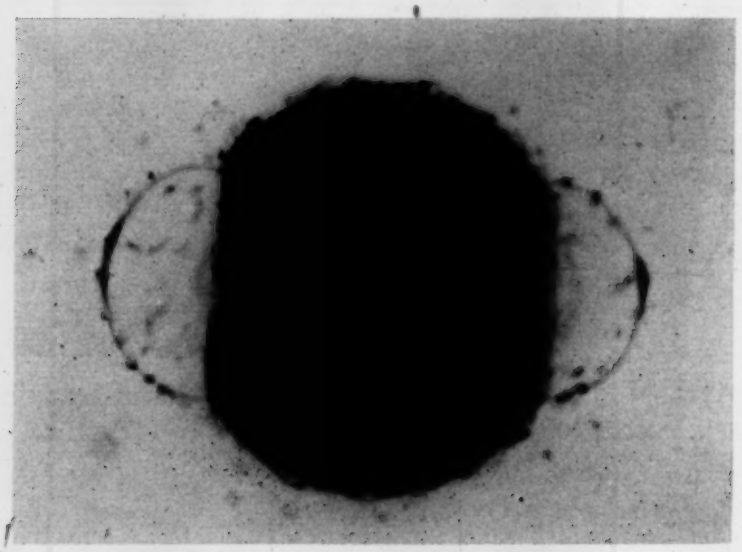

Fig. 4. A dispermic egg with two sperm-entry marks. ×*ca.* 400

2. Experiments with urethane-treated eggs

According to SUGIYAMA (1956), unfertilized eggs narcotized with urethane can be fertilized without breakdown of the cortical granules. In order to test if the mark can be produced even when the granular breakdown is inhibited, some experiments were performed with urethane-treated eggs.

Unfertilized eggs were exposed to urethane-sea water (50 ml of 1 M urethane plus 50 ml of sea water) for 3 minutes, washed in ordinary sea water and then inseminated. 32 to 45 seconds after insemination they were put into the Ca-rich solution. Neither breakdown of the cortical granules nor formation of the sperm-entry mark occurred. This result indicates that the breakdown of the cortical granules is one of the factors necessary for the for-

mation of the mark.

3. Relation between the third factor and formation of the mark

MOTOMURA (1950) reported that a substance extractable from sea urchin eggs with acidic sea water is an indispensable factor for hardening of the fertilization membrane. The substance was called the third factor. In order to study the relation between the third factor and formation of the mark, the following experiments were carried out.

By the method described by MOTOMURA (1950), unfertilized eggs were exposed to acidic sea water of pH 4.5 for 20 minutes, followed by two washings with ordinary sea water. 32 to 45 seconds after insemination, the eggs were removed into the Ca-rich solution, but no formation of the mark occurred.

As the next step, pH of the acidic sea water which had been used in the above procedure was adjusted to 8.2 by adding NaOH. This medium is sea water containing the third factor. Eggs previously extracted with acidic sea water were inseminated in this medium. 32 to 45 seconds after insemination, the eggs were transferred into the Ca-rich solution including the third factor.* In contrast to the result of the former experiment, the mark was formed.

According to MOTOMURA (1954), when eggs treated with sodium sulfite-sea water are inseminated, the membrane formation occurs without hardening, being due to inactivation of the third factor in the eggs. In the present experiment, eggs were treated with sodium sulfite-sea water (20 ml of 10/27 M Na_2SO_3 plus 80 ml of sea water) for 30 minutes, and then inseminated. No mark was formed when they were removed in the Ca-rich solution.

4. Failure of formation of the mark in a mixture of 10/27 M $CaCl_2$ and sea water

When inseminated eggs were put into a mixture of $CaCl_2$ and sea water (10 ml of 10/27 M $CaCl_2$ plus 90 ml of sea water), no formation of the mark occurred. In this case, although numerous minute particles appeared in the perivitelline space near the point

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* Unfertilized eggs were extracted with acidic isotonic salt solution (pH 4.5) for 30 minutes, the pH of which was adjusted to 8.2 and then 10/27 M $CaCl_2$ was added in ratio of 10 : 1.

of sperm-entry, they failed to attach to the membrane. The particles gradually aggregated and converted into rod-like flocculates.

In the experiments with the mixture of 10/27 M $CaCl_2$ and the isotonic salt solution, such a formation of the rods did not take place, while the mark was always produced on the fertilization membrane. It is known that in normal fertilization, soon after the full elevation of the fertilization membrane, the rods occasionally appear in the perivitelline space near the point of sperm-entry, which are considered to be the same as those observed in the mixture of $CaCl_2$ and sea water.

Discussion

It is well-established that the hardening of the fertilization membrane, which begins soon after the elevation, is completed in a few minutes, as the result of adhesion of the substance derived from the cortical granules with the participation of Ca and the third factor (Hobson 1932, Sugiyama 1938 a and b, Motomura 1941, 1950 and 1957, Runnström 1948, Runström, Monné and Wicklund 1944 and 1946, Runnström and Wicklund 1950, Endo 1952 and 1954).

Fig. 5 shows the time-relation among the hardening of the membrane, the secretion of the third factor and the formation of the sperm-entry mark. It may be seen that when fertilized eggs in the stage III are transferred to the Ca-rich solution 32 to 45 seconds after insemination, the formation of the mark proceeds as shown in the figures A to G. During this period, the membrane is only partly elevated and is scarcely hardened. When eggs are transferred into the Ca-rich solution before or after this period (stages II and IV), no formation of the mark occurred. This means that the formation of the mark is closely related to the hardening process of the membrane.

When eggs are narcotized with urethane, the mark fails to be formed, fertilization taking place without breakdown of the cortical granules. Therefore, it may be said that the cortical granules may participate in the formation of the mark.

When the mixture of $CaCl_2$ and sea water is used, no mark is formed, but rods appear in the perivitelline space. In the case of the Ca-rich salt solution, the mark is formed but no rods appear,

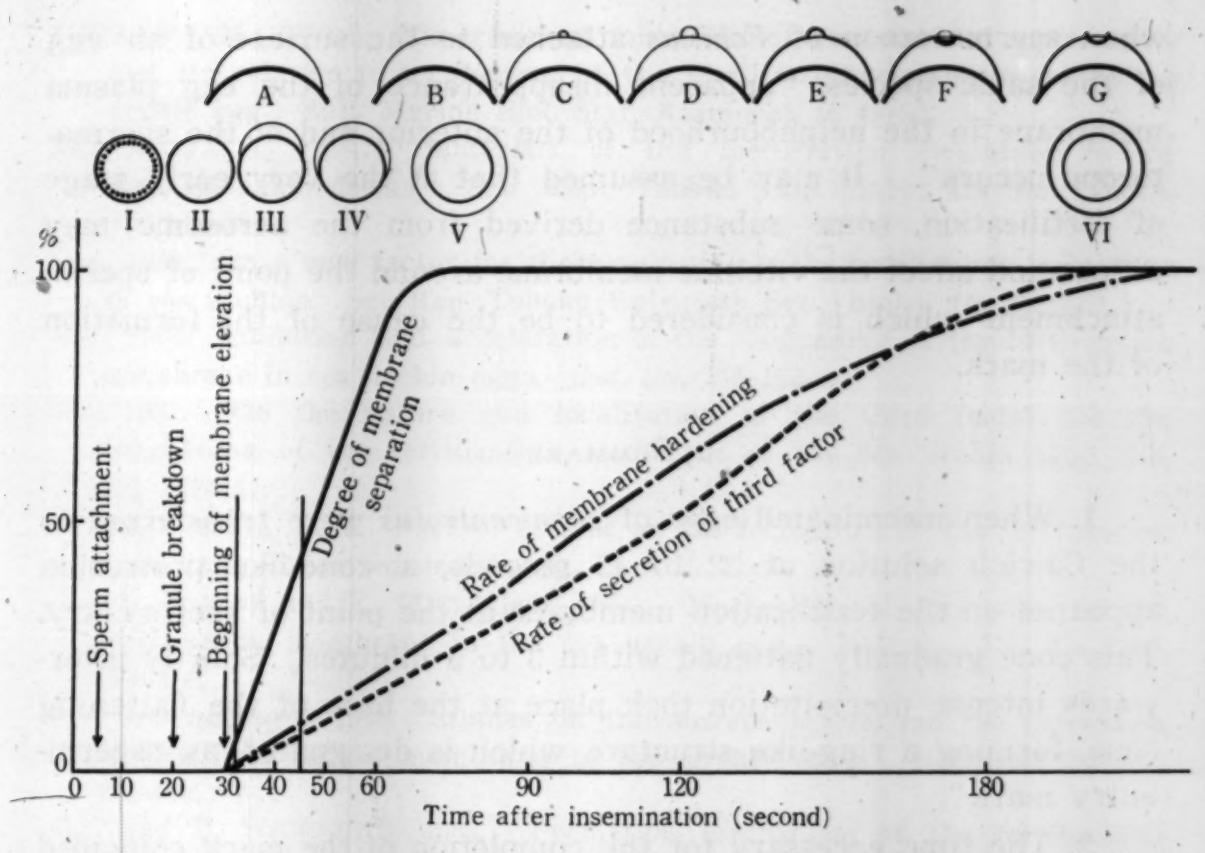

Fig. 5. Time-relation among formation of the sperm-entry mark, hardening of the membrane and secretion of the third factor.
A—G: Stages of the formation of the sperm-entry mark.
I—VI: Stages of the membrane formation in ordinary sea water.
Curves of the membrane hardening and the third factor secretion were adopted from SUGIYAMA (1938 a) and MOTOMURA (1950) respectively. The details are described in the text.

suggesting that there might be a inverse relation between the rods and the mark.

MOTOMURA (1950) reported that the secretion of the third factor reaches a maximum at 3 to 5 minutes after fertilization. As shown in Fig. 5, the formation of the mark is completed at the same time as the secretion of the third factor reaches a maximum. If the third factor is removed from eggs using acidic sea water, the eggs lose their capacity to form the mark. Such eggs, however, can produce the mark, if the third factor is supplemented. Sulfite-treatment of eggs before fertilization also results in a failure of the formation of the mark. From these facts, the third factor is considered to be necessary for the formation of the mark.

ROTHSCHILD (1956) demonstrated by an electron micrograph that

when spermatozoon of *Echinus* attached to the surface of an egg of the same species, "apparent disappearance of the egg plasma membrane in the neighbourhood of the anterior end of the spermatozoon occurs". It may be assumed that at the very early stage of fertilization, some substance derived from the acrosome may diffuse and affect the vitelline membrane around the point of sperm-attachment which is considered to be the cause of the formation of the mark.

Summary

1. When inseminated eggs of *Hemicentrotus* were transferred to the Ca-rich solution at 32 to 45 seconds, a cone-like protrusion appeared on the fertilization membrane at the point of sperm-entry. This cone gradually flattened within 3 to 5 minutes. Shortly afterwards intense precipitation took place at the base of the flattening cone, forming a ring-like structure which is designated as "sperm-entry mark".

2. The time necessary for the completion of the mark coincided with that of a maximum secretion of the third factor, the last of which is indispensable for the membrane hardening. The eggs which had been deprived of the third factor failed to form the mark, but such eggs produced the mark if they were immersed in the Ca-rich solution including the third factor.

3. It was suggested that the formation of the mark may be due to regional difference in nature of the membrane between the limited area around the point of sperm-entry and the remaining area.

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THE DIFFERENTIATION *IN VITRO* OF THE ISOLATED ENDODERM IN THE PRESENCE OF THE NEURAL FOLD IN *TRITURUS PYRRHOGASTER*

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INTRODUCTION

Although a piece of the endoderm isolated from the *Triturus* gastrulae or neurulae is not capable of differentiation, it gives rise to definite endodermal tissues if combined with a piece of the mesoderm (OKADA, 1954, 1955 a, b, 1957, 1960, TAKATA, 1960). The results of combination of various regions of endoderm and mesoderm indicate that the type of the endodermal tissue obtained depends upon the mesodermal areas added as well as upon the prospective significance of the isolated endoderm. Moreover, the age of the endoderm donor exerts an influence upon the differentiation.

That the neural crest material is also able to enhance the differentiation of the neurula endoderm into the pharynx has been shown by OKADA (1957, 1960). However, it is still an open question whether there exists a regional morphogenetic difference in the neural fold on the endodermal differentiation, or an age difference in the competence of endodermal areas. In the present paper, the differentiation of the isolated endodermal areas combined with the neural fold areas is studied systematically by the explantation experiments using the *Triturus* embryos. The explants were cultured

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long enough to allow identification of the individual endodermal tissues.

Material and Methods

The analyses in the present investigations were carried out exclusively on the embryos of *Triturus pyrrhogaster*. The endodermal pieces to be explanted were excised from the superficial layer of the endoderm as shown in Figure 1. The beginning gastrulae (Okada and Ichikawa's Stage 11) were used to cut out the piece, *e 1*, lying dorsally to the pigmented groove, which is the first indication of the invagination groove, and the piece, *e 3*, lying at the center of the vegetal unpigmented hemisphere. From the middle neurulae (St. 17, St. 18), four different endodermal pieces were taken, *i.e.*, the anterior region, *aE 1*; the antero-ventral region, *vE 1*; the middle region, *E 2;* and the posterior region, *E 3*. According to the fate map of *Triton taeniatus* projected by Balinsky (1947) and that of *Triturus pyrrhogaster* projected by Nakamura and Tahara[3] (oral communications), the piece, *e 1*, includes the presumptive pharynx, stomach and liver, the piece, *e 3*, the presumptive intestine, the piece, *aE 1*, the presumptive pharynx and stomach, the piece, *vE 1*, the presumptive stomach and liver, and the pieces, *E 2* and *E 3*, the presumptive intestine.

The neural fold material was cut out from two regions of the neurula (Sts. 17–19), namely, the head neural fold and trunk neural fold. Included in the explant was the part of the neural plate adjoining directly to the neural fold (Fig. 1). In embryos at stages 17 to 19, the neural crest material is still a part of the primary ectoderm, retaining its epithelial character. The migration of crest elements begins later at the early tail-bud stage (Ichikawa, 1937). The mesoderm underlying the neural fold was not included in the explant.

The isolated endoderm was combined with the neural crest material and inserted between two epidermal pieces stripped off from the antero-ventral region of the epidermis covering the

[3] A communication at the 25th Annual Meeting (1955) of the Zoological Society of Japan and personal communications.

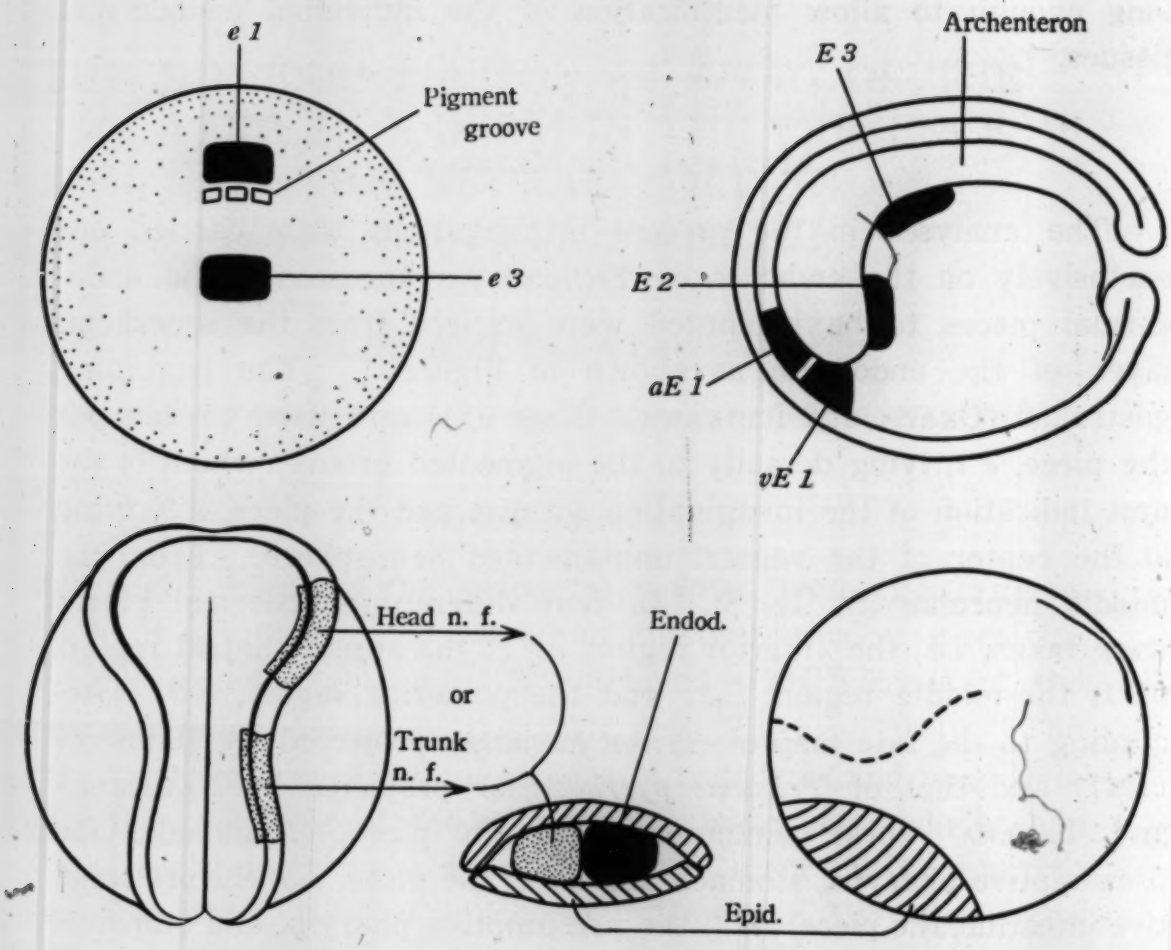

Fig. 1. Scheme of operation showing the endodermal areas and neural fold areas excised from the *Triturus* embryos and explants. Description in text.

mesoderm-free area of the latest gastrula (St. 15). Using aseptic precautions, operation and cultivation were done in HOLTFRETER's solution adjusted to pH 7.3–7.4 with sodium bicarbonate or Tris. The explants were cultured at 18°C. for 35–75 days, during which time the yolk platelets within the endodermal cells have almost completely disappeared. For microscopical observations, the explants were fixed in BOUIN's solution, sectioned at 10 microns and stained with MAYER's hemalum and picroblauschwarz. The histological identification of the endodermal tissues in the present experiments was based on the criteria described in the previous report (TAKATA, 1960). When the endodermal piece from the beginning gastrula and middle neurula was enclosed in two epidermal pieces without addition of mesodermal pieces and cultured as long as possible, no

sign of differentiation was noticed under the present culture conditions (TAKATA, 1960).

RESULTS

Series 1. Explantation of the neural fold in the absence of the endoderm. To test the differentiation capacity of the neural fold without the endodermal piece, each piece of the neural fold was enclosed in the epidermis, and cultured for about 15 days at 18°C. The explants rounded into balls with the melanophores on the surface.

In the series with the head neural fold, a rather large amount of ectomesenchyme frequently appeared within the explants. Further, the neural tissue was contained in most explants but the balancers were formed in only a few cases. Besides, a thin layer of mesothelium was occasionally developed.

In the series with the trunk neural fold, ectomesenchyme was found very frequenly, though in a very limited amount. The neural tissue was encountered in the relatively low percentage. Some of the explants produced mesothelium. In agreement with NIU's results (1947), the amount of melanophores differentiated by the trunk neural fold was larger than that produced by the head neural fold.

Series 2. Explantation of endoderm plus head neural fold. Pieces from six endodermal regions of the gastrula (St. 11) and neurula (St. 17, St. 18), namely, *e 1*, *e 3*, *aE 1*, *vE 1*, *E 2* and *E 3*, were reared in the epidermis with the head neural fold. The explants developed into rather expanded vesicles in about three weeks. But thereafter some of the explants lost the internal pressure and became corrugated. Such condition often led to extrusion of the endodermal piece. The results of histological observation are summarized in Table 1.

Ectodermal components and neural crest derivatives. In most of the explants, the neural tissue, ectomesenchyme, melanophores and xanthophores were encountered. Besides, the mesothelium was found in only a very small number of cases. In some series, the balancers or cartilage was infrequently revealed.

Table 1. Differentiation of tissues in the explants of the endoderm combined with the head neural fold

Isolated endoderm:	Gastrula		Neurula			
	e 1	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants:	45	58	32	41	58	33
No. of explants retaining the explanted endoderm:	43 (95%)	33 (57%)	29 (90%)	37 (90%)	30 (52%)	8 (24%)
No. of explants containing:						
Pharynx	38	11	26	21	6	1
Gill pouches	21	3	14	5	1	0
Oesophagus	1	1	2	1	1	0
Stomach	1	1	2	2	1	0
Liver	0	0	5	4	0	0
Intestine	1	6	2	4	7	3
Pancreas	0	0	0	2	0	0
Unidentifiable endodermal tissue	0	1	0	0	1	0
Undifferentiated endoderm	7	18	4	16	17	4
Neural tissue	41	29	27	29	26	8
Ectomesenchyme	42	22	29	37	30	8
Mesothelium	6	5	0	12	5	0
Melanophores	43	29	28	33	30	8
Xanthophores	36	18	27	18	28	7
Cartilage	8	0	8	8	0	0
Balancers	1	1	1	2	0	0
Notochord	1	0	0	0	1	0
Muscle	5	0	0	0	0	0
No. of explants with any differentiated endodermal tissues:	40 (89%)	18 (31%)	27 (84%)	23 (56%)	15 (26%)	4 (12%)

Mesodermal component. The muscle was found in 5 explants from the series with piece *e 1*. This may be explained by the fact that the endodermal piece, *e 1*, was taken from an area close to the prechordal plate, which is known to give rise to the muscle in explantation (OKADA and HAMA, 1943).

Endodermal components. In the series with the gastrula endoderm, piece *e 1* differentiated into the pharynx in a very high percentage, accompanied frequently by gill pouches, and piece *e 3* into the pharynx or the intestine. In the seris with the neurula endoderm, pieces *aE 1* and *vE 1* differentiated at very high frequencies into the pharynx equipped sometimes with gill pouches (Fig. 2). The same endodermal areas produced the oesophagus, stomach, and intestine, at a very low frequency respectively. In the series with piece *vE 1*, a small piece of pancreatic tissue was found in the

Fig. 2. A pharyngeal (right, below) and gill epithelium (left, above) within ectomesenchyme formed in an explant of the endodermal piece, *aE 1*, and head neural fold. Cartilage (middle, above) is also found embedded in ectomesenchyme. *ca.* ×310

vicinity of the intestinal tissue. On the contrary, in the explants containing the presumptive intestine (*E 2* or *E 3*), a high frequency of intestine was observed. The liver was rarely found and only in pieces *aE 1* and *vE 1*.

Series 3. Explantation of endoderm plus trunk neural fold. It was very difficult to culture the explants long enough, especially in the series with the endoderm including the presumptive intestine, namely, *e 3*, *E 2* and *E 3*, because of the tendency for the endodermal piece to be extruded from the epidermal jacket. The results of microscopic examination are summarized in Table 2.

Ectodermal components and neural crest derivatives. Most of the explants produced the neural tissue, ectomesenchyme, melanophores and xanthophores. The balancers, cartilage, and mesothelium were also formed in some explants.

Table 2. Differentiation of tissues in the explants of the endoderm combined with the trunk neural fold

Isolated endoderm:	Gastrula		Neurula			
	e 1	*e 3*	*aE 1*	*vE 1*	*E 2*	*E 3*
No. of available explants:	47	59	41	15	64	44
No. of explants retaining the explanted endoderm:	36 (77%)	37 (63%)	26 (63%)	12 (80%)	20 (31%)	21 (48%)
No. of explants containing:						
Pharynx	21	1	20	5	0	0
Gill pouches	14	0	9	0	0	0
Oesophagus	0	0	1	1	0	0
Stomach	0	2	3	1	0	0
Liver	0	0	2	3	0	0
Intestine	2	6	2	3	0	1
Pancreas	1	1	1	0	0	0
Unidentifiable endodermal tissue	0	0	0	1	1	0
Undifferentiated endoderm	14	29	4	5	19	20
Neural tissue	34	25	19	10	7	13
Ectomesenchyme	36	33	26	12	17	21
Mesothelium	8	5	5	6	2	1
Melanophores	35	29	26	11	18	21
Xanthophores	25	14	25	8	10	8
Cartilage	8	0	3	1	0	0
Balancers	1	0	1	0	0	0
Notochord	0	1	0	0	0	1
Muscle	7	4	1	0	1	0
No. of explants with any differentiated endodermal tissues:	23 (49%)	8 (14%)	23 (56%)	8 (53%)	1 (2%)	1 (2%)

Mesodermal component. In the series with piece *e 1*, the muscle was infrequently found scattered in ectomesenchyme.

Endodermal components. In the series with the endodermal material from the gastrula, piece *e 1* frequently differentiated into the pharynx often provided with gill pouches, but infrequently into the intestine and pancreas. On the other hand, piece *e 3* mainly produced the intestine, which was sometimes associated with the pharynx, stomach, and pancreas. In the series with the endoderm isolated from the neurula, piece *aE 1* mostly gave rise to the pharynx and gill pouches, while piece *vE 1* formed the pharynx and intestine (Fig. 3) at moderate frequencies. The oesophagus, stomach, liver, and pancreas were produced only rarely. Pieces *E 2* and *E 3* hardly showed tissue differentiation.

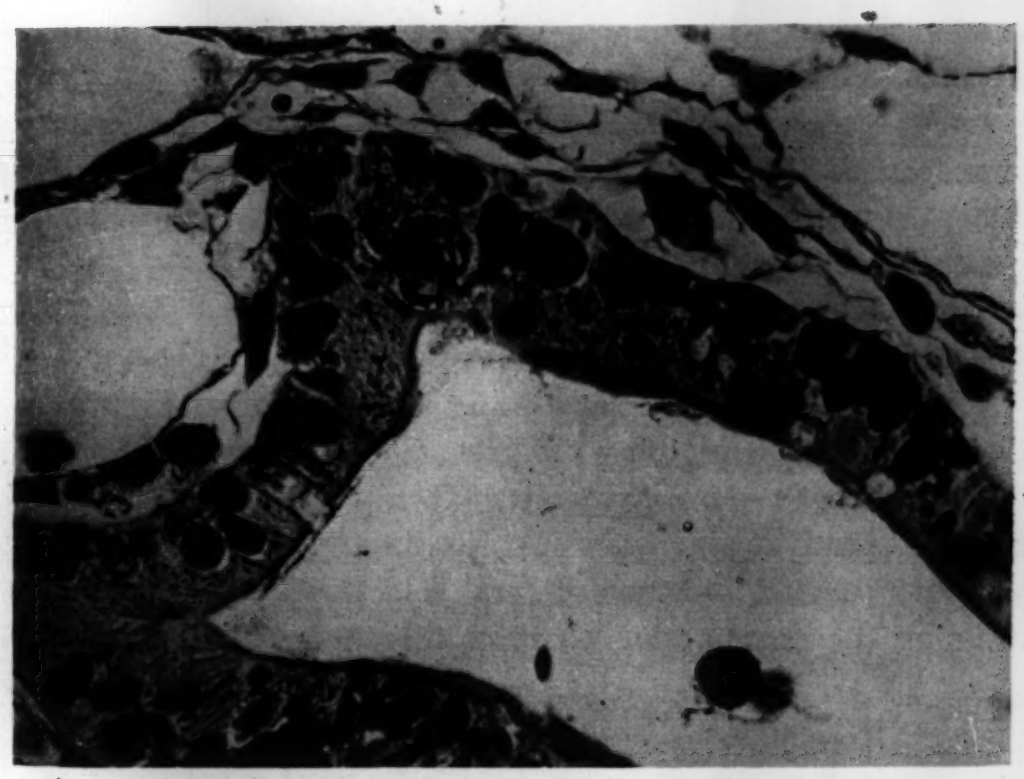

Fig. 3. An intestinal epithelium within ectomesenchyme formed in an explant of the endodermal piece, *E 3*, combined with the head neural fold. Note the goblet cells and cuticular border of the epithelium. *ca.* ×340

Conclusions and Discussion

In the present investigations addition of the neural fold to the endodermal pieces was found to elicit various types of endodermal differentiation such as pharynx, oesophagus, stomach, liver, intestine, and pancreas. This confirms Okada's (1957, 1960) results that the isolated endoderm differentiated into the definite endodermal tissues under the influence of neural crest material, though the differentiation into the pancreas was lacking in his experimental series. Moreover, the present data indicated a clear difference in the frequency of successful differentiation of the explants between various series: The explants with the anterior endodermal piece showed a frequency of differentiation higher than the explants with the posterior endodermal piece, and the presence of head neural fold was more favorable for the endodermal differentiation than the presence of trunk neural fold (cf. Table 1 and Table 2). The suspension of differentiation was caused by extrusion of the endo-

dermal piece from the explant, the endodermal piece alone being unable to differentiate (OKADA, 1957, 1960, TAKATA, 1960). That the extrusion occurred more frequently in combination with neural fold-endoderm than in combination with mesoderm-endoderm (TAKATA, 1960) may reflect a difference in the affinities between these embryonic areas.

From Tables 1 and 2, it is obvious that the most frequent type of endodermal differentiation is represented by the pharynx. This has also been confirmed by OKADA. As seen in Figure 4, the endodermal piece containing the presumptive pharynx differentiated into the pharynx more frequently than the endodermal piece of other prospective significance. No difference in morphogenetic effects of the head and trunk neural fold was found between series containing the presumptive pharynx (Fig. 4). On the other hand,

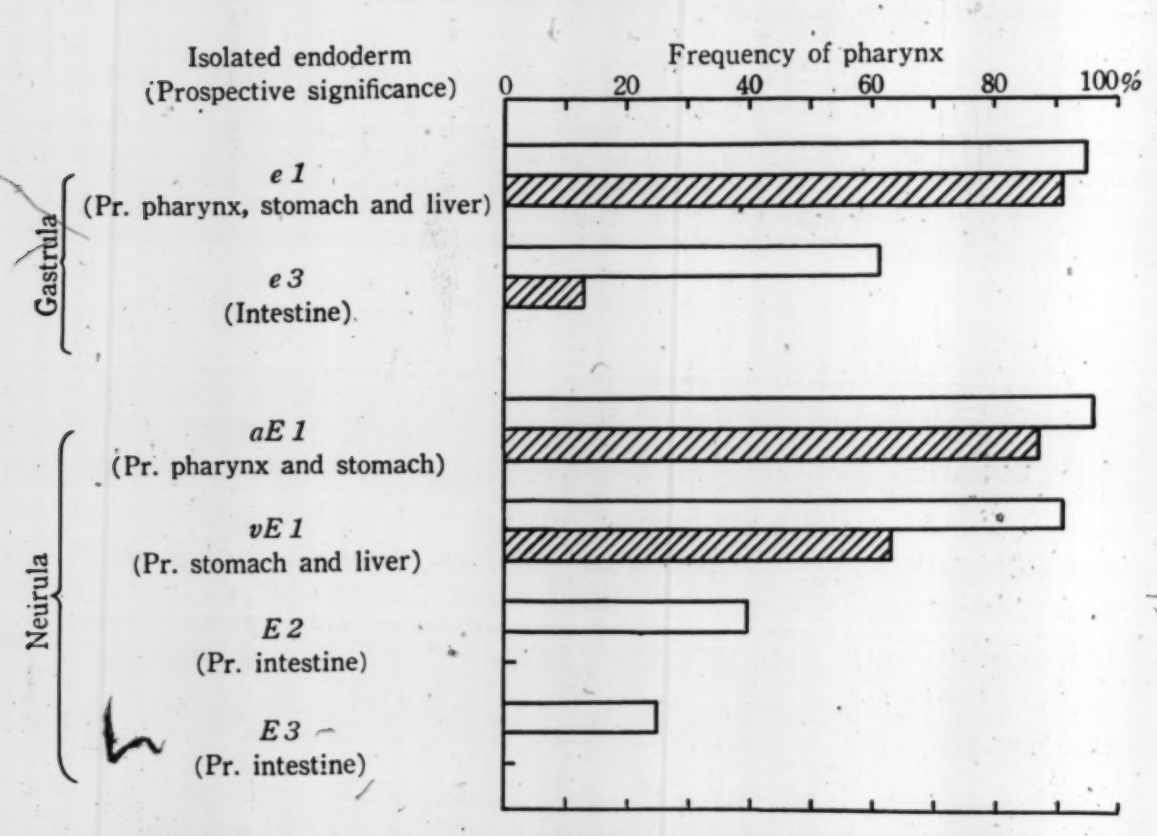

Fig. 4. Diagrams showing the pharyngeal differentiation in the series of the head neural fold (blank bars), or of the trunk neural fold (shaded bars). The frequency of pharynx is expressed by the percentage of the number of explants containing the pharynx against the number of explants with any differentiated endodermal tissues in each series.

in the series with the endodermal piece of other prospective significance, the frequencies of pharynx were higher in combination with the head neural fold than in combination with the trunk neural fold. The stronger enhancement of the pharynx differentiation by the head neural crest may be linked to the larger amount of mesenchyme produced by the head neural crest as compared with the amount of mesenchyme produced by the trunk neural crest. All the pharynges found in the explants were surrounded with ectomesenchyme. It therefore seems reasonable to assume that mesenchyme of the neural fold origin favors the development of pharynx, like mesenchyme of the mesodermal origin whose importance for the pharyngeal differentiation has been established.

Other types of differentiation of the isolated endoderm were the oesophagus, stomach, liver, intestine, or pancreas. Their frequencies were, however, very low. Of some interest is the fact that the frequency of intestine against the explants with any differentiated endodermal tissues was higher in the series with the presumptive intestine than in the series with other endodermal areas. Those intestines were found either enclosed in the mesothelium or embedded in ectomesenchyme. The liver tissue was found in the series with pieces *aE 1* and *vE 1* from the neurula combined either with the head neural fold or with the trunk neural fold.

The differentiation of head or trunk neural fold is represented by ectomesenchyme, cartilage, melanophores, and xanthophores. As Table 1 shows, the cartilage was formed in the series of the anterior endodermal piece combined with the head neural fold. This is in good conformity with the experiments of HÖRSTADIUS and SELLMAN (1945) and of E. W. OKADA (1955) showing the dependence of cartilage formation on the presence of the anterior endoderm. Also in the series of the anterior endoderm combined with the trunk neural fold, some cartilages were observed (Table 2). However, in no explants with the posterior endoderm cartilage formation occurred. Further, the explants of neural folds without endodermal piece failed to realize cartilage formation. All these facts lead to the conclusion that under the influence of the anterior endoderm, ectomesenchyme is able to differentiate cartilage at least in the present experimental conditions.

Summary

1. The endodermal pieces containing the different prospective significance were cut out of the beginning gastrulae and middle neurulae of *Triturus pyrrhogaster*, combined with the head neural fold or the trunk neural fold from the middle neurulae, and cultured for 35–75 days at 18°C.

2. Under the influence of neural fold material, the isolated endoderm was able to differentiate into the definite endodermal tissues such as pharynx, oesophagus, stomach, liver, intestine, and pancreas. The anterior endodermal piece tended to differentiate into the pharynx, and the posterior endodermal piece into the intestine.

3. The pharynx differentiatiation of the anterior endodermal piece was favored by the presence of the neural fold, no difference in this effect being observed between the head and trunk neural fold. However, the pharynx differentiation of pieces taken from other regions was more frequent in the series with the head neural fold than in the series with the trunk neural fold.

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EXPERIMENTAL STUDIES ON THE ADRENAL CORTEX OF THE MOUSE-FETUS

I. EFFECTS OF MATERNAL ADRENALECTOMY ON THE ADRENAL OF THE FETUS BASED ON HISTOLOGY AND VOLUME DETERMINATION

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INTRODUCTION

It has been shown that adrenalectomy of pregnant rats results in a hypertrophy of the fetal adrenal near the end of pregnancy (INGLE and FISHER 1938, WALAAS and WALAAS 1944, DAVIS and PLOTZ 1954, KNOBIL and BRIGGS 1955, JOST, JACQUOT and COHEN 1955, CHRISTIANSON and JONES 1957). Removal of the fetal hypophysis by decapitation in the rat retarded the growth of the adrenal and this retardation in decapitated fetuses could be prevented by injection of ACTH (WELLS 1948, 1949, KITCHELL and WELLS 1952). The suggestion has further been made that maternal ACTH probably does not pass through the placental barrier under physiological conditions (JOST 1953, JONES, LLOYD and WYATT 1953). These various observations have led to the assumption that the hypertrophy of the fetal adrenal following maternal adrenalectomy does not result from the elevated level of maternal ACTH, but from that of fetal ACTH (JOST 1957). However, it is of importance to know whether or not this hypertrophy can be produced in other species, since, in the dog, ROGOFF and STEWART (1927) reported that maternal adrenalectomy caused no enlargement of the fetal adrenal.

It is probable that during the course of development, the fetal adrenals pass through a period of receptivity for stimulating factors. If possible, detection of such a critical period may be of a great interest. The present study was undertaken to know whether the fetal adrenal of the mouse does enlarge as a consequence of maternal

adrenalectomy, and, if so, at which stage this enlargement could be detected. In addition, the neonatal loss of size of the adrenal was observed.

Materials and Methods

From 39 pregnancies, 179 fetuses were collected for the use in this study. In addition, 48 neonatals of ages ranging from 1 to 7 days were used. All of the pregnant females and neonatals were albinos of the bc strain inbred from the Japanese native mouse (Kondo, Himeno, Ikoma and Katsuragi 1953). Females were placed with males overnight and examined for copulation plugs next morning. In this strain, most pregnant females bring forth their offsprings on the 20th day, so that the 19th day is regarded as a day before parturition. Occasionally, parturition does not occur on the 20th day. Thus, seven fetuses on the 20th day of pregnancy were available for comparison with the newborns on the same day. Pregnant females were divided into five main categories according to the age of pregnancy when they were sacrificed, and subdivided into 2 or 4 experimental groups each category respectively.

(I) Sacrificed on the 15th day of pregnancy
- C. Non-operated
- E. Adrenalectomized on the 11th day of pregnancy

(II) Sacrificed on the 16th day of pregnancy
- C. Non operated
- E. Adrenalectomized on the 12th day of pregnancy

(III) Sacrificed on the 17th day of pregnancy
- C. Non-operated
- E. Adrenalectomized on the 13th day of pregnancy

(IV) Sacrificed on the 18th day of pregnancy
- C. Non-operated
- E. Adrenalectomized on the 14th day of pregnancy

(V) Sacrificed on the 19th day of pregnancy
- C_1. Non-operated
- C_2. Sham-adrenalectomized on the 15th day of pregnancy
- E_1. Adrenalectomized on the 15th day of pregnancy
- E_2. Adrenalectomized on the 11th day of pregnancy

Bilateral adrenalectomy was performed under ether anesthesia via the dorso-lumbar approach. Satisfactoriness of the operation was carefully examined at autopsy under a dissecting microscope. Some pregnant mice (Category V, group C_2) received bilateral sham-operations on the dorsolateral lumbar wall by an incision of the mid-dorsal skin.

Fetal adrenals were removed together with kidneys, and the left gland was fixed in BOUIN's fluid, and the right in 10% formalin. The formalin-fixed material was frozen and sectioned at 20 μ, and was stained with Sudan black B for observation of lipid distribution. The BOUIN-fixed material was embedded in paraffin, sectioned at 10 μ, and stained with hematoxylin-eosin for histological and volumetric studies. The volume of the gland was determined by the paper-weight method. Outline of every 4th section was traced with the aid of a camera lucida on the ledger paper.

The volumes of the left adrenals from the fetuses on the 20th day of pregnancy and from the neonatals of ages of 1, 2, 3, 5 and 7 days after birth were measured in the same way. The adrenal volume was expressed both in mm^3 and in the ratio of the adrenal volume to the body weight. In addition, nuclear count per unit area was made in the cortex and medulla of the adrenal on the 19th and 20th day of pregnancy as well as at birth. These determinations were to establish change in cell size at birth.

RESULTS

A. The normal growth of the adrenal

Volumes of the adrenal in the fetuses from non-operated mothers and of neonatals are shown in Table 1. On the 15th day, the medullary elements were in contact with the cortical gland dorso-medially and little of them were found therein. On the 16th day, most medullary elements migrated into the cortical tissue, scattering as small islets. Owing to this migration of medullary elements, a great difference in adrenal volume was observed between the 15th and the 16th day. From the 16th to the 18th day, the rate of increase in adrenal volume was slower than that of increase in body weight, so that the ratio, adrenal volume/body weight, declined gradually. On the other hand, from the 18th to the 19th day, since

Table 1. Average volumes of left adrenal of fetal (F) and neonatal (N) mouse from non-operated mothers

Age (days)	No. of mouse	Body wt. ±S.D.** (g)	Adr. vol. ±S.D.** ($mm^3 \times 10^2$)	Adr. vol. ($mm^3 \times 10^2$) / Body wt. (g) ±S.D.**
F 15	11	0.17±0.023	1.9±0.51	11.6±2.75
F 16	14	0.40±0.046	5.5±0.96	13.5±3.26
F 17	15	0.50±0.015	5.9±0.87	12.2±1.54
F 18	12	0.69±0.074	6.4±1.14	9.3±1.10
F 19	25	0.94±0.081	9.3±1.82	9.8±1.51
F 20	7	1.22±0.05	10.0±0.34	8.2±0.37
N 1*	13	1.18±0.26	9.0±1.14	7.3±0.87
N 2	9	1.34±0.27	9.3±1.60	6.9±0.79
N 3	9	1.73±0.27	9.7±1.55	6.1±1.02
N 5	9	2.10±0.24	10.5±1.30	5.4±0.50
N 7	8	2.66±0.23	12.1±3.00	4.7±0.87

* Newborns on the 20th day of pregnancy. F 20 and N 1 are on the same age.

** Standard deviation $\left(\sqrt{\frac{d^2}{N-1}}\right)$

The difference between the absolute adrenal volumes of F 19 and those of N 1 was insignificant statistically, but the difference between F 20 and N 1 was significant ($Pt<0.05$).

growth of the adrenal exceeded that of the body growth, the ratio increased a little. The average adrenal volume on the first day of life was somewhat smaller than that on the 19th day of pregnancy, the difference being statistically insignificant. However, since the adrenals of the fetuses on the 20th day of pregnancy (F 20) were significantly larger than those of the newborns of the same day (*i.e.* the first day of life, N 1). This may indicate a probable involution of the gland through parturition which will be discussed later. Because of a steady increase of the body weight in contrast to the involution of the gland, the ratio of the adrenal volume to the body weight fell markedly on the first day of life from that on the 19th day of pregnancy. In the medulla, the average number of nuclei per unit area was almost constant on the 19th and 20th day of pregnancy and on the first day of life (Table 2). In the cortex, the average nuclear number on the first day of life was found to be greater than those on the 19th and 20th day of pregnancy in spite of the involution (Table 2). Thus, it is suggested that the neonatal involution of the adrenal was owing to the shrinkage of cortical cells, although the degeneration and disappearance of cells

Table 2. Average number of cell nuclei per unit area in the adrenal medulla and cortex of fetuses and of neonatals. Counts of 10 areas were made per animal

Age (days)	No. of mouse	Av. No. of medullary cell nuclei/unit area*	Av. No. of cortical cell nuclei/unit area**
F 19	12	17.0±1.2***	63.1±4.6***
F 20	7	16.0±1.6	57.6±4.5
N 1	13	15.6±1.2	66.8±3.2

* 713 μ^2

** 4449 μ^2

*** Standard Deviation.

Table 3. Data from fetuses in mothers adrenalectomized 4 days prior to sacrifice, and comparison with those from fetuses in non-operated mothers

Categories	Day of pregnancy on which data were taken	groups*	No. of litters	No. of fetuses observed	Body wt. ±S.D.** (g)	Left adrenal volume: Av. ±S.D.** ($mm^3 \times 10^2$)	Left adrenal volume: Vol. ($mm^3 \times 10^2$) / Body wt. (g) ±S.D.**	Pt***	
I	15	C	3	11	0.17±0.023	1.9±0.51	11.6±2.75	0.8	
		E	3	12	0.17±0.028	2.0±0.52	11.9±1.89		
II	16	C	3	14	0.40±0.046	5.5±0.96	13.5±3.26	0.7	
		E	4	13	0.26±0.034	3.6±0.67	14.1±1.88		
III	17	C	3	15	0.50±0.015	5.9±0.87	12.2±1.54	0.3	
		E	3	18	0.46±0.082	5.4±0.88	11.5±1.52		
IV	18	C	3	12	0.69±0.074	6.4±1.14	9.3±1.10	0.01	
		E	3	16	0.53±0.059	6.0±1.19	11.3±2.70		
V	19	C_1	5	25	0.94±0.081	9.3±1.82	9.8±1.51	0.8	0.001
		C_2	3	18	0.96±0.075	9.3±1.69	9.7±1.60	0.001	
		E_1	5	19	0.79±0.100	9.2±1.61	11.5±0.99		
		E_2	1	6	0.56±0.180	6.6±1.03	11.9±1.23		

* C, C_1; fetuses in non-operated mothers. C_2; fetuses in sham-operated mothers. E, E_1; fetuses in adrenalectomized mothers. E_2; fetuses in mother adrenalectomized on day 11.

** Standard deviation is calculated by the formula; $\sqrt{\frac{d^2}{N-1}}$

*** The difference between E and C in the ratio of adrenal volume to body weight was determined by the "t" test. Pt below 0.05 is significant, especially Pt below 0.001 is highly significant.

were not encountered histologically. Development of the adrenal after birth was very slow, and the ratio, adrenal volume/body weight, declined day by day.

B. Effects of maternal adrenalectomy on the growth of adrenals

Volumetric data are presented in Table 3, and the average intensity of the sudan reactions in the fetal adrenal cortex is shown in Table 4.

15th day of pregnancy (*Category I in Table 3*). General histological findings in the E adrenals were almost similar to those in

Table 4. Average intensity of the sudan reactions in the adrenal cortex of the fetuses from adrenalectomized and non-operated mothers

mother	day of pregnancy → / zone ↓	15	16	17	18	19
non-operated	outer	−	+	++	++	++
	intermediate	−	+	+	++	+++
	inner	−	+	++	+++	++++
adrenalecto-mized 4 days before	outer	−	+	+	+	++
	intermediate	−	±	±	±	±
	inner	−	±	+	++	++

outer: Corresponding to adult zona glomerulosa.
intermediate: Corresponding to adult outer z. fasciculata.
inner: Corresponding to adult inner z. fasciculata and z. reticularis.
− no reaction.
± weak reaction in some cells.
+ weak reaction in all cells.
++ moderate reaction.
+++ strong reaction.
++++ intense reaction.

the C adrenals. The parenchyma of the cortex was divided into irregular cell masses or cords, the cells of which were indistinctly outlined, having strongly eosinophilic cytoplasms and asymmetric nuclei. No difference in the fetal body weight and in the adrenal volume was observed between C and E. It is concluded that maternal adrenalectomy had hardly any effects on the fetal adrenal at this stage.

16th day of pregnancy (*Category II in Table 3*). The cells of the outer zone of the cortex were relatively small, packed closely and grouped as ill-defined arcuated masses by fine connective tissue fibers. Medullary elements were scattering as small islets, many of which were rather centralized in the gland, dividing the cortical tissue into reticular branched cords.

In the E adrenals, the cells in the intermediate zone were rather clearly outlined. The cytoplasm was more eosinophilic; sinusoids between the cell cords were somewhat spacious in comparison with C. The fetal body weight and the adrenal volume were both less in E than in C, but adrenal volume/body weight remained the same between E and C.

In sections stained with Sudan black B (Figs *a*-1 and *b*-1), the adrenals both in E and in C revealed the cortex stained faintly. In the outer zone, fine lipid droplets were scattered in the cells. In the intermediate zone, few sudanophilic materials were observed in the E adrenals. Since the fetal growth was slightly retarded in E, the paucity of sudanophilic materials in the E adrenals may not necessarily be the result of maternal adrenal deficiency. It is concluded that effects of maternal adrenalectomy on the fetal adrenal were still hardly noticed on the 16th day of pregnancy.

17th day of pregnancy (*Category III in Table 3*). The difference in histological pattern, based on hematoxylin-eosin staining, was still not evident between the E and the C adrenals. In the intermediate zone, the cells were large and were indistinctly outlined, being arranged in rather irregular cords. The cytoplasm was granular, eosinophilic and somewhat vacuolated. In the E adrenals, the cortical cells seemed to be somewhat larger and more distinctly outlined. Since the fetal body weight and adrenal volume in E were both inferior to those in C, adrenal volume/body weight was approximately the same in the E and C fetuses. In the E adrenals, the cortex was less sudanophilic than that in C (compare Fig. *a*-2 with Fig. *b*-2).

18th day of pregnancy (*Category IV in Table 3*). The histological patterns of the E and C adrenals were almost identical. The cortical cell cords became straight. Since the fetal body weight was smaller in E than in C, the ratio, adrenal volume/body weight, was significantly larger in E than in C. In the normal growth, the ratio fell markedly from the 17th day to the 18th day (see F 17 and F 18 in Table 1). Considering this trend of the adrenal growth, it would be unable to be regraded as significant change that the E fetuses, which were smaller in body weight than the C fetuses, were higher in the ratio, adrenal volume/body weight, than the C

fetuses. Sudanophilic lipid droplets in the C adrenals (Fig. *a*-3) were coalesced in the outer zone, and tended to be finer and more numerous towards the inner portion of the gland. The sudan stainability of the E adrenals (Fig. *b*-3) was inferior to that of the C adrenals. Especially in the intermediate zone, only a few fine droplets were found, the sudan stainability being much weaker than the C adrenals on the 17th day (compare Fig. *b*-3 with Fig. *a*-2). Of two possible explanations for the decreased stainability *i.e.*; depletion by active secretion and a retarded accumulation, the former is more likely.

19th day of pregnancy (*Category V in Table 3*). The cells in the outer zone of the C_1 adrenals enlarged and became more distinctly organized into the small whorls. The intermediate zone was composed of cords of cells with a marked radial arrangement. The cells were large, having a relatively sharp cell limit and vacuolated eosinophilic cytoplasm. In the fetuses from sham-operated mothers (C_2), the histological pattern of the adrenals was almost similar to that of C_1. In the E_1 adrenals, the outer zone was very lightly stained, whereas the other part was remarkably eosinophilic. In the intermediate zone, the cells were large, having a strongly eosinophilic cytoplasm with weak vacuolation. The sudan stainability was almost the same in the C_1 and C_2 adrenals. In the intermediate zone of the E_1 adrenals, quite different from the C_1 adrenals, sudanophilic droplets were extremely fine and scanty (compare Fig. *a*-4 with Fig. *b*-4). It seems likely that some secretion in the E_1 adrenals occurred.

There was little difference in the body weight between the C_1 and the C_2 fetuses. On the other hand, the body weight was smaller in E_1 than in both C_1 and C_2. Since the absolute adrenal volume in E_1 was almost equal to that in C_1 and C_2, the ratio of the adrenal volume to the body weight was much larger in E_1 than in both C_1 and C_2, the difference being highly significant. In the normal growth, the ratio rose from the 18th to the 19th day (see F 18 and F 19 in Table 1). Considering this trend of the adrenal growth, it was highly significant that the fetal adrenals in E_1 enlarged in proportion to the fetal growth. It is concluded that maternal adrenalectomy acted on enlargement of the fetal adrenal on the

19th day of pregnancy. The E_2 fetal adrenals were considered also to have significantly enlarged under a lack of the maternal adrenals.

Discussion

1. The neonatal involution of the adrenal

The loss of size of the adrenal after birth has been described in various mammals, and especially, in man, an immense literature has been accumulated on the subject (Scammon 1926, Benner 1940, Swinyard 1943). Josimovich, Ladman and Deane (1954) reported that in rats, the average volume of adrenal cortex was smaller in the ½ day neonatals than in the 21½ day fetuses. They said, "This decrease in cortical volume appeared to be the result of two factors: (1) a conspicuous shrinkage of the cells of the fasciculata and reticularis; and (2) the disappearance of cortical cells lying among the medullary clusters." They further stated, "The most likely explanation of this cortical involution seems to us to be a decline in pituitary activity after birth. Another possibility, less probable on the basis of current information, is that the decline reflects the removal of maternal adrenotropin, small quantities of which may cross the placenta." In regard to the involution of the human adrenal after birth, on the basis of the assumption that the "fetal cortex" is maintained by the fetal hypophysis which is forced to release LH by estrogen of maternal origin, it has been emphasized that the involution results from the cessation of maternal estrogen (Benirschke, Bloch and Hertig 1956).

The data obtained from the present study showed that adrenal involution at birth occurred also in mice. The shrinkage of the cortical cells was observed, but disappearance of cells was not confirmed. Maternal ACTH in the rat is said not to pass through the placenta (Jost 1953, Jones, Lloyd and Wyatt 1953). If this be the case in mice, the neonatal shrinkage of the cortex should primarily be due to the decline of the pituitary activity of the youngs, which may, in turn, suggest the interruption of the stimulation of the piuitary gland of the young by maternal hormones. Concerning this problem, more detailed studies are required.

2. Effect of maternal adrenalectomy on the fetal adrenal

The present investigation gives a new evidence that the fetal

adrenal glands in mice enlarge following adrenalectomy of the mother. This enlargement was demonstrated in the significantly higher ratio of the adrenal volume to the body weight on the 19th day of pregnancy, in comparison with the normal. On the 18th day, some increase in the ratio was observed, but this was not considered to be significant.

The fetal adrenal enlargement in the adrenalectomized rat has been interpreted as the following; the decrease of maternal corticosteroids in the fetal circulation raises the activity of the fetal pituitary which causes the fetal adrenal enlargement (JONES, LLOYD and WYATT 1953, DAVIS and PLOTZ 1954, JOST, JAQUOT and COHEN 1955, CHRISTIANSON and JONES 1957). This interpretation has been based on several lines of facts such as the increase in the fetal adrenals after maternal adrenalectomy can only be obtained as long as the fetal pituitary is present and also such as there is an increase in fetal adrenal weight relative to body weight after maternal adrenalectomy as well as hypophysectomy and adrenalectomy (CHRISTIANSON and JONES 1957). Considering the result obtained by RAYNAUD (1959) that in the mouse-fetuses whose hypophyses were destroyed by X-rays, the adrenal reduced its size, indicating a capacity of the fetal hypophysis stimulating on the adrenal growth. The interpretation in the rat could be perhaps applied also to the case of the mouse.

It is of considerable interest to know at what time endocrine secretion first begins in the fetal glands. In the present experiment, no significant enlargement of the glands was observed until the 19th day of pregnancy after removal of the maternal adrenals. This observation is closely related to the finding that hypertrophy of mitochondria and the first appearance of ascorbic acid in the glands occurred coincidently on the 19th day of pregnancy (EGUCHI and HASHIMOTO 1957), suggesting that during the prenatal life, the adrenal cortex of the mouse is most active on the 19th day. Furthermore, after maternal adrenalectomy, lipid droplets became extremely fine and scanty in the intermediate zone of the fetal adrenal cortex on the 18th and the 19th days of pregnancy. This finding suggests that secretion took place to some extent. The critical period of the onset of secretory activity is probably between the 18th and the 19th day of pregnancy. However, it is, of course,

another question whether the fetal adrenals act similarly under the normal physiological condition. The results obtained showed an activation of the fetal adrenal only after maternal adrenalectomy.

Summary

Changes in histology, volume and lipid distribution in the adrenals of the fetuses after maternal adrenalectomy in the mouse were investigated. In addition, the growth of the adrenals during fetal and early postnatal stages was studied.

From the 16th to the 18th day, the rate of increase in adrenal volume was slower than that of increase in body weight, while, from the 18th to the 19th day, the situation was reversed. From the 19th day of pregnancy to the first day of life, the adrenal volume declined slightly, while, that of fetuses on the 20th day of pregnancy was even larger than those of neonatals of the same day (the first day of life). Thus, neonatal loss of the adrenal size occurs in mice.

Maternal adrenalectomy resulted in little change in the fetal adrenals until the 18th day of pregnancy. On the 18th and especially on the 19th day, the ratio, adrenal volume/body weight, of the fetal adrenals from adrenalectomized mothers was larger than that from normal mothers.

In microscopical examinations, all the adrenals of experimentals revealed somewhat less sudanophilia than the controls. Especially on the 18th and the 19th day, sudanophilic droplets were extremely minute and scanty in the intermediate zone of the cortex of the experimentals.

From the above findings, it is concluded that maternal adrenalectomy in mice enlarged the fetal adrenals on the 19th day of pregnancy, and activated them for secretion from about the 18th day.

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EXPLANATIONS OF PLATE 1

Photomicrographs of sections of the adrenals stained with Sudan black B, taken by the use of a 10×ocular and a 4 mm objective. All were cut at 20 μ. All figures are oriented with the capsule to the top, the medulla to the bottom. Figures in the upper row (*a*) are the adrenals of the fetuses from the normal mothers, figures in the lower row (*b*) the adrenals of the fetuses from the adrenalectomized mothers. Magnification ×345.

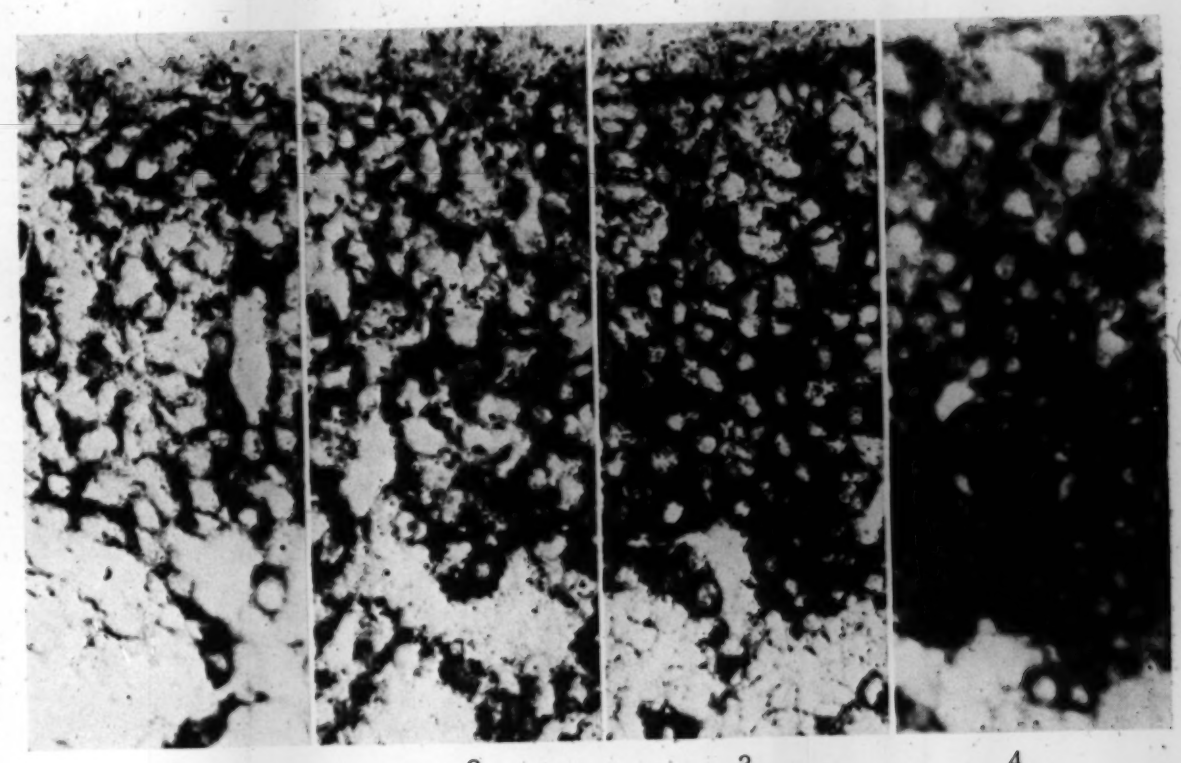
Figs. *a*-1 and *b*-1. Fetus on day 16 of pregnancy. The outer zone is loaded with sudanophilic materials. The remainder of the cortex is less sudanophilic in Fig. *b*-1 than in Fig. *a*-1.

Figs. *a*-2 and *b*-2. Fetus on day 17 of pregnancy. In Fig. *a*-2 all zones are moderately sudanophilic. Particularlly towards the inner zone, lipids increase in amount. In Fig. *b*-2, lipids are slightly scanty.

Figs. *a*-3 and *b*-3. Fetus on day 18 of pregnancy. Normal adrenal shows an increase in the content of lipids. The inner part of the cortex is rather intensely colored. The entire cortex is less sudanophilic in Fig. *b*-3 than in Fig. *a*-3. Especially in the intermediate zone in Fig. *b*-3, only a few fine droplets scatter.

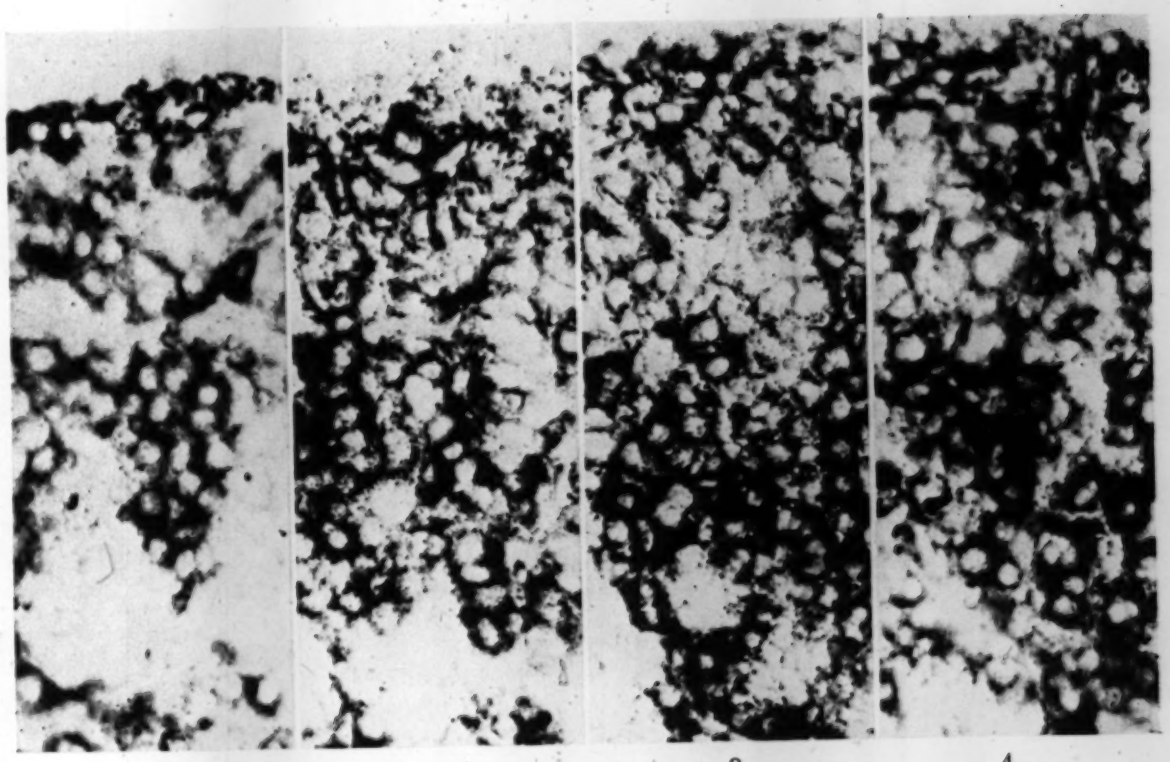
Figs. *a*-4 and *b*-4. Fetus on day 19 of pregnancy. The lipids are heavily accumulated in Fig. *a*-4. Entirely, the lipids are somewhat less in Fig. *b*-4 than in Fig. *a*-4. Note the intermediate zone in Fig. *b*-4 where the lipid materials are extremely scanty.

a



1 2 3 4

b



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